Cell cycle-based mechanism of epigenetic centromere propagation

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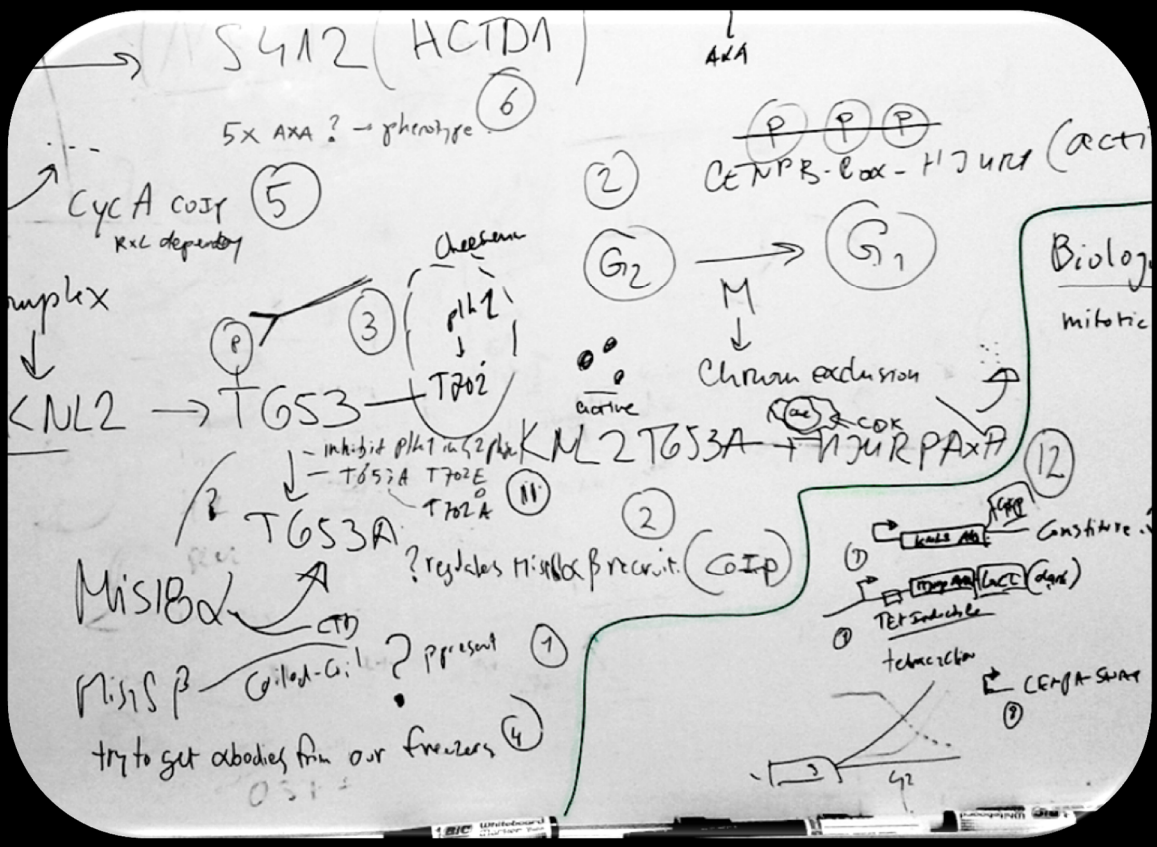

Dissertation presented to obtain the Ph.D degree in Molecular Biology

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
April, 2017

UNIVERSIDADE NOVA DE LISBOA

Драги мама и тата, надам се да сте поносни на мене колико сам ја на вас. Данас сам овде захваљујући вама и вашој подршци. Волим вас пуно и вама посвећујем ову тезу.

Ана

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Research work coordinated by:

FUNDAÇÃO CALOUSTE GULBENKIAN
Instituto Gulbenkian de Ciência

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Declaration

I declare that this dissertation and the data presented are the result of my own work, as developed between 2012 and 2017 in the laboratory of Dr. Lars Jansen at the Instituto Gulbenkian de Ciência in Oeiras, Portugal. Specific author contributions are indicated in each chapter, in the Acknowledgements section.

Financial support was granted by Fundação para a Ciência e a Tecnologia, doctoral fellowship SFRH/BD/51878/2012 and ERC-2013-CoG-615638–Epimechanism.

Declaração

Declaro que esta dissertação de doutoramento e os dados nela apresentados são o resultado do meu trabalho, desenvolvido entre 2012 e 2017 no laboratório do Dr. Lars Jansen no Instituto Gulbenkian de Ciência em Oeiras, Portugal. As contribuições de cada autor são indicadas em cada capítulo na secção dos Agradecimentos/Acknowledgements.

O apoio financeiro foi concedido pela Fundação para a Ciência e Tecnologia, através da bolsa de doutoramento SFRH/BD/51878/2012 e por fundos do Conselho Europeu de Investigação (ERC) ERC-2013-CoG-615638–Epimechanism.

Summary

The centromere is a chromosomal locus that is responsible for nucleating a structure called the kinetochore during mitosis. This structure serves as a binding platform for microtubules during mitosis, enabling segregation of genetic material to two daughter cells. Although fascinating, the function of the centromere was not the focus of my PhD work. Rather, it was the epigenetic nature of its propagation. The centromere is epigenetically marked and inherited through incorporation of a specialized H3 variant called CENP-A. CENP-A is necessary and sufficient for specification of centromere genomic location and its inheritance. Incorporation of CENP-A into centromeric nucleosomes is orchestrated by several factors, and occurs in a cell cycle dependent manner, upon mitotic exit. Previous work from our laboratory demonstrated that the key molecular switch is driven by Cyclin-dependent kinases 1 and 2 (Cdk1/2) that negatively control the timing of CENP-A incorporation. Brief inhibition of these kinases resulted in precocious centromeric incorporation of CENP-A. This led to a proposal where the CENP-A loading machinery is present and poised for CENP-A assembly, but is held inactive due to Cdk1/2 activities. Key proteins necessary for the process of CENP-A deposition include the Mis18 complex and the CENP-A specific chaperone HJURP, which bears CENP-A-specific nucleosome assembly activity.

In Chapter 2, I describe how negative Cdk1/2 control is exerted upon members of the Mis18 complex. By identifying a key phosho-residue on the largest member of this Mis18 complex, M18BP1, we demonstrate that Cdk1/2 are controlling M18BP1 centromere localization, rather than its activity. Mutating key phospho-residue in M18BP1 resulted in premature centromere recruitment of this protein, thus demonstrating a direct

involvement of Cdk1/2 in regulating M18BP1 centromere targeting. However, premature centromere recruitment of the Mis18 complex did not result in complete alleviation of inhibition of CENP-A assembly.

In Chapter 3, I focus on the Cdk1/2 dependent regulation of the HJURP chaperone. As in the case of the Mis18 complex centromere localization, Cdk1/2 are acting directly upon HJURP centromeric targeting. Functional inhibition of HJURP is exerted at the level of its centromeric localization, rather than activity, given that mutations in key Cdk-dependent phosho-residues of HJURP result in premature localization of HJURP. Furthermore, these mutations result in a low level of precocious nascent CENP-A assembly.

In Chapter 4, we show that simultaneous uncoupling of the CENP-A loading factors, M18BP1 (chapter 2) and HJURP (chapter 3) from cell cycle control results in a full recapitulation of CENP-A assembly under high Cdk activities, indistinguishable from G1 assembly. This indicates that these two assembly factors are the main targets of the cell cycle control mechanism, restricting CENP-A assembly to G1 phase.

In summary, this work expands and provides direct evidence for the previously recognized role of Cdk1/2 in regulation of inheritance of epigenetic centromere. We define a dual inhibitory mechanism that is sufficient to maintain cell cycle restricted centromere propagation and characterize the molecular mechanism of how CENP-A assembly is turned on and subsequently turned off.

Sumário

O centrómero consiste numa região do cromossoma que é responsável pela agregação do cinetocóro. Esta estrutura actua como uma plataforma na qual os microtúbulos se ligam durante o processo de mitose, permitindo a distribuição do material genético pelas duas células-filhas. No entanto, embora fascinante, o assunto central desta tese não é a função do centrómero, mas sim a natureza epigenética da sua propagação.

O centrómero é epigeneticamente definido e herdado através da incorporação de uma variante especializada da histona H3 designada por CENP-A. Esta proteína é necessária e suficiente não só para a determinação da localização genómica do centrómero, como também para sua propagação às células-filhas. A incorporação da CENP-A nos nucleossomas centroméricos é organizada por diversos factores e depende da fase do ciclo celular, ocorrendo especificamente durante a saída da mitose. Resultados prévios do laboratório tinham já mostrado que o controlo da incorporação de CENP-A é regulado negativamente pelas Cinases Dependentes de Ciclina 1 e 2 (Cdk1/2). Dado que a inibição temporária destas cinases resultou numa deposição precoce de CENP-A no centrómero, foi proposto que a maquinaria responsável por esta incorporação estaria já presente e pronta para funcionar, mas que seria mantida inactiva devido à actividade das Cdk1/2. Outras proteínas essenciais no processo de deposição da CENP-A incluiriam o complexo Mis18 e a chaperona HJURP, esta última que demonstrou ter uma actividade específica para a deposição da CENP-A durante a formação dos nucleossomas do centrómero.

O Capítulo 2 descreve a forma como o controlo negativo das Cdk1/2 é exercido sobre os membros do complexo Mis18. Através da identificação

de uma fosforilação-chave num resíduo no maior dos membros do complexo Mis18, M18BP1, demonstrou-se que as Cdk1/2 controlam a localização centromérica de M18BP1 - e não a sua actividade. A mutação deste fosfo-resíduo em M18BP1 resultou no recrutamento prematuro desta proteína, o que revelou que as Cdk1/2 têm um efeito directo na regulação da localização de M18BP1 para o centrómero. No entanto, este recrutamento prematuro do complexo Mis18 não resultou na atenuação completa da inibição deste sobre a incorporação de CENP-A no centrómero.

No Capítulo 3, a regulação da chaperona HJURP pelas Cdk1/2 é discutida. De forma semelhante ao controlo da localização centromérica do complexo Mis18, as Cdk1/2 actuam directamente sobre a localização centromérica de HJURP. A inibição da função desta chaperona, mais uma vez, ocorre ao nível da sua localização - e não da sua actividade - dado que mutações em fosfo-resíduos dependentes de Cdk1/2 em HJURP são suficientes para induzir baixos níveis de incorporação precoce de CENP-A.

No Capítulo 4 é demonstrado que o desacoplamento simultâneo dos factores que regulam a acumulação de CENP-A, M18BP1 (Capítulo 2) and HJURP (Capítulo 3), do ciclo celular resulta numa recapítulação completa da incorporação de CENP-A sob altos níveis de actividade de Cdk, o que é idêntica à deposição desta histona na fase G1.

Em resumo, este trabalho aumenta e reforça o importante papel já previamente reconhecido das Cdk1/2 na regulação da propagação epigenética do centrómero. Este estudo não só define um mecanismo inibitório duplo que revelou ser suficiente para restringir a propagação do centrómero à fase apropriada do ciclo celular, como também caracteriza o

mecanismo molecular pelo qual a deposição de CENP-A no centrómero é activada e inactivada.

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Lars, you are an amazing and inspiring supervisor. Thank you for patiently sitting next to me and teaching me everything from how to clone to how to write a scientific paper. Thank you for putting so much trust in me and letting me develop my own ideas as well as represent our lab on multiple conferences. My journey as a scientist doesn't end here, but the foundation that you have set will always be a part of my scientific endeavors. I am privileged to have you as my role model not only for on how the science should be done, but also how to deal with ups and downs of everyday life. I could not have asked for a better supervisor. Thank you!

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Let´s start!

List of Publications

In chronological order:

Ana Stankovic and Lars E.T. Jansen (2013). Reductionism at the vertebrate kinetochore, *J. Cell Biol*. *200*, 7–8.

Ana Stankovic, Lucie Y. Guo, João F. Mata, Dani L. Bodor, Xing-Jun Cao, Aaron O. Bailey, Jeffrey Shabanowitz, Donald F. Hunt, Benjamin A. Garcia, Ben E. Black and Lars E.T. Jansen (2017). A Dual Inhibitory Mechanism Sufficient to Maintain Cell-Cycle-Restricted CENP-A Assembly. *Molecular Cell*, *65*, 231–246.

Ana Stankovic and Lars E.T. Jansen (2017). Quantitative microscopy reveals centromeric chromatin stability, size and cell cycle mechanisms to maintain centromere homeostasis*, Springer series, ´´Progress in Molecular and Subcellular Biology``, In press*

CHAPTER 1

General Introduction:

Cell cycle and Centromeres

This chapter contains sections of the publication: Ana Stankovic and Lars E.T. Jansen (2017). Quantitative microscopy reveals centromeric chromatin stability, size and cell cycle mechanisms to maintain centromere homeostasis. Springer series, ´´Progress in Molecular and Subcellular Biology``, *In press*

1.1 The cell cycle

Cell reproduction is conveyed by an orderly sequence of events in which cell duplicates its contents and divides in two. This cycle of duplication and divisions is known as the cell cycle.

The process of duplication occurs in in S or synthesis phase, which is followed by equal partition of the duplicated material between two daughter cells during mitosis (M phase). S phase is flanked by two phases in which the cell continues to grow. The G1 phase (Gap1) is the time period between the completion of M phase and the beginning of S phase (Figure 1.1). During G1 phase, initial steps for DNA replication are taken in form of DNA ´´licencing´´, consisting of the formation of a specific protein-DNA complex called the pre-replicative complex (pre-RC) (Blow and Dutta, 2005; Machida et al., 2005). When cells are committed for division they enter the S phase (Synthesis phase). At this stage, the initiation step of DNA replication occurs, and consists of activation of the pre-RC complex (assembled at G1 phase) and in establishment of bidirectional replication forks, leading to DNA replication and chromosome duplication (Bell and Dutta, 2002; Tanaka and Araki, 2010). Once DNA is replicated, cells enter the second Gap (or G2) phase. In this phase, the cell increases in size and activates regulatory mechanisms that will ensure the cell´s competence for mitotic entry (Pollard et al., 2017) (Figure 1.1). In Mitosis, the duplicated chromosomes along with organelles are segregated and equally divided to two daughter cells. The first stage of mitosis, prophase, is characterized by nuclear envelope breakdown, followed by the onset of DNA condensation together with centrosome-mediated microtubule nucleation that will form the mitotic spindle. As microtubules emanate from the spindle poles, they contact the kinetochore on each chromosome. Simultaneously, continuous

growth of microtubules at their plus-end positions chromosomes close to the centre of the mitotic spindle. Once all chromosomes are aligned with sister chromatids facing opposite poles (bi-orientation), cell will progress from metaphase into anaphase. During anaphase, two sister chromatids are separated towards the opposite poles of the cells. Finally, in telophase, the nuclear envelope is reformed together with formation of the contractile ring which constricts the cell equator during cytokinesis to give rise to two daughter cells (Pines, 2006; Pollard et al., 2017).

1.2 Cell-cycle control system and Cyclin-dependent kinases

To ensue faithful and one-directional flow of the cell cycle, eukaryotic cells possess a complex network of regulatory proteins known as the cell-cycle control system (Crosby, 2007; Morgan, 1997; Nurse, 2000; Pollard et al., 2017). This system guarantees that the events of the cell cycle will occur in a sequential manner and that each process has been completed before the next one begins. The central components of the cell cycle control system are a family of enzymes called cyclin-dependent kinases (Cdks) that catalyze the covalent attachment of ATP-derived phosphate groups to Serine or Threonine of their protein substrates. This phosphorylation modulates substrate's enzymatic activity and interaction with other protein complexes. Importantly, Cdk activities cyclically rise and fall as cell progress through cell cycle. These oscillations result in cyclical changes in phosphorylation of components of the cell-cycle machinery, driving the transition between cell cycle stages. Switching Cdk activities on and off at the appropriate times is partly the responsibility of another set of proteins in the control system—the cyclins. The binding of the enzymatic kinase with the corresponding cyclin results in the formation of a cyclin-Cdk complex,

which is followed by phosphorylation of Cdk by an activating protein kinase (CAK) at a conserved threonine residue (Krek and Nigg, 1992; Morgan, 2007; Solomon et al., 1993). Without cyclins, Cdk bear little enzymatic activity. Cyclins can be classified into four categories depending on the timing of their accumulation: the G1 phase cyclin (cyclin D in vertebrates), the G1/S phase cyclin (cyclin E in vertebrates), the S phase cyclin (cyclin A in vertebrates) and the mitotic cyclin (cyclin B in vertebrates) (Morgan, 2007) (Figure 1.1). The concentration of cyclins oscillates during the cell cycle and their abundance is regulated at several levels: transcriptional, translational and at the level of protein stability. One of the common ways of regulating cyclin accumulation is achieved via control of their nuclear import and export; cyclin E and A are present in the nucleus during interphase (import is favoured over export) whereas cyclin B is cytoplasmic in interphase and enters the nucleus only upon mitotic entry (Murray, 2004). At the end of mitosis, ubiquitin-mediated proteolysis drives the destruction of mitotic Cyclin B, which results in inactivation of Cdks activities (Morgan, 2007; Zachariae et al., 1998). This inactivation allows cell to re-enter interphase. Upon mitotic exit, low Cdk activities allow the recruitment of components of pre-replicative (pre-RC) complex that will serve as a landing pad for the assembly of other proteins known to be essential for the initiation of DNA replication. Formation of pre-replicative complex (pre-RC) allows chromatin to be ´´licenced`` for replication in subsequent S phase. As cell advances from G1 into S phase, the activities of S phase-specific Cdks are raising, and together with cdc7 kinase, activate pre-RCs that recruit DNA replicating enzymes on sites of DNA replication. Therefore, differential levels of Cdk activities are essential for temporal disconnection between licencing and initiation of DNA replication; while the licensing step requires low Cdk activity, the initiation step needs

high Cdk activity (Arias and Walter, 2007; Pollard et al., 2017; Tanaka and Araki, 2010).

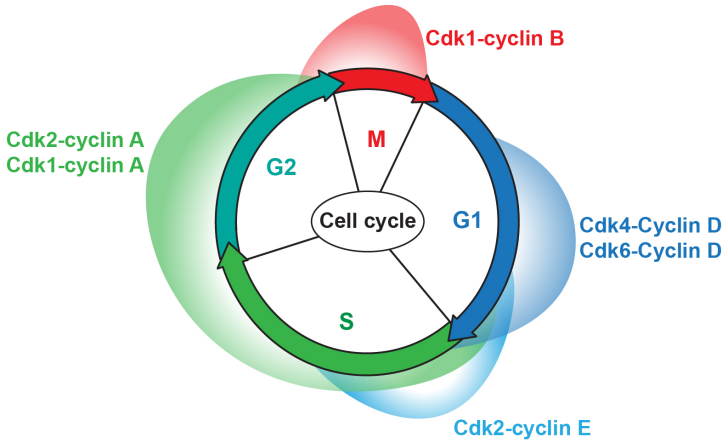

Figure 1.1 Overview of eukaryotic cell cycle stages together with principal (human) cyclin-Cdk complexes that are active at a specific phase. There are the four stages of the cell cycle, the G1 (Gap 1), S, G2 (Gap 2) and M (Mitosis) phases (Nurse, 2000; Pollard et al., 2017). Processes and transitions between cell cycle phases are controlled by cyclin-Cdk complexes. In G1 phase the major complex is formed between Cdk4-cyclin D and Cdk6-cyclin D, S phase entry is controlled by Cdk2-cyclin E, Cdk2-cyclin A and Cdk1-cyclin A regulate the completion of S and G2 phases, and Cdk1-cylin B control mitosis.

1.3 Cyclin-dependent kinases and their choice of substrates

Once activated through binding to its regulatory Cyclin subunit, the active site of Cdks recognize and phosphorylate Serine or Threonine present on a substrate, which are embedded within a typical [S/T*]-P-X-[K/R] consensus motif (where X is any amino acid), and K/R amino acid is not absolutely required) (Errico et al., 2010; Morgan, 2007; Murray, 2004). However, due to the ubiquitous occurrence of Ser/Thr–Pro sequences in both substrates and non-substrates, Cdk targets can harbour an additional cyclin docking ´´Cy`` motif (Morgan, 2007). For example, both cyclin A and cyclin E

contain a hydrophobic binding pocket on their surface, that recognizes an RXL motif (where X is any amino acid) on a Cdk substrate (Brown et al., 2007). This motif is important to increase substrate specificity, especially when the substrate contains a truncated Cdk consensus motif (S/T-P). RXL motifs are prevalent in S phase substrates of Cdk2-cyclin A and Cdk2-cyclin E and in some CKI proteins (Errico et al., 2010; Morgan, 2007). Whether there is an equivalent, different motif for cyclin B is unknown, however, Cyclin B shows little affinity towards RXL motifs due to the different sequence in its hydrophobic patch compared to the one present in Cyclin A/ Cyclin E (Brown 2007). The most pervasive mechanism by which cyclin B confers substrate specificity is most likely through its subcellular localization.

1.4 Centromere function

Centromeres are specialized genomic loci that drive accurate genome segregation across cell divisions. The core region of the centromere provides a structural platform for formation of the kinetochore, a protein complex that links chromosomes to spindle microtubules during mitosis (Figure 1.2) (Cheeseman and Desai, 2008; Foltz et al., 2006; Okada et al., 2006). The centromere nucleates the kinetochore via the assembly of a large group of proteins, the centromere-associated network (CCAN) (Cheeseman and Desai, 2008; Foltz et al., 2006; Izuta et al., 2006). Members of the CCAN network are constitutively present at the centromere throughout the cell cycle. During mitosis, they recruit a secondary protein complex known as the kinetochore. The core microtubule binding site of the kinetochore is comprised of the conserved microtubule-binding KMN network, consisting of the protein KNL1 as well as the Mis12 and Ndc80 complexes (Cheeseman et al., 2004, 2006; DeLuca et al., 2006).

The KMN network serves as an initial scaffold for the recruitment of Spindle assembly checkpoint proteins (SAC). SAC is the mitotic checkpoint ensuring that anaphase does not take place before chromosomes are bi-oriented on the spindle (facing opposite poles of the mitotic spindle) (Musacchio and Salmon, 2007). The SAC achieves this by generating a signal at unattached kinetochores, known as the MCC complex (MAD2, BUBR1, BUBR3 and CDC20). The MCC complex directly inhibits the anaphase promoting complex (APC/C), which is the key regulator of anaphase onset. In this way, unattached kinetochores generate a stop signal allowing for the spindle to attach. Upon spindle attachment, the MCC signal generation is inhibited, in part, by stripping off the MCC components from the kinetochore (Musacchio and Salmon, 2007). While the checkpoint blocks anaphase as long as kinetochores are unattached and not bi-oriented, regulation of such proper kinetochore-microtubule attachments is achieved by an error-correction mechanism and its key effector, Aurora B (Carmena et al., 2012). Inter-centromere localized Aurora B acts by phosphorylating its kinetochore targets and de-stabilizing attached microtubules (Figure 1.2). Upon correct attachment, kinetochore geometry changes due to the exerted tension. This shift removes Aurora B form its substrates, thus stabilizing the microtubule attachments that generate sufficient tension. As bi-orientation is a state in which the highest amount of tension will be exerted on kinetochore pairs, this state is preferably stabilized (Dewar et al., 2004; Watanabe, 2012).

The broader centromeric domain, termed pericentric heterochromatin, is the site which keeps the chromosomes together during mitosis. This is enabled by a ring-like molecule called cohesin which encompasses replicated DNA strands upon their duplication in S phase (Figure 1.2) (Haarhuis et al., 2014). Cohesin is dynamically associated along the length

of the chromosome; however most of the cohesin is unloaded during prometaphase, leaving only centromeric cohesin to keep the chromosomes together (Waizenegger et al., 2000). Cohesin degradation along with Cyclin B degradation marks the onset of anaphase. This simultaneous degradation is orchestrated by the APC/C. APC directly targets Cyclin B for degradation (Pines, 2006). Additionally, APC degrades securing (Uhlmann et al., 1999), which is an inhibitor of Separase. Separase, in turn, is a protease that cleaves cohesion at the metaphase to anaphase transition (Nasmyth and Haering, 2009; Peters et al., 2008), ensuring rapid segregation of chromosomes, coinciding with Cyclin B degradation and mitotic exit.

1.5 Centromere organization across different species

The role of the centromere in driving chromosome segregation is highly conserved, yet centromere size and genomic localization is remarkably different across eukaryotes (Malik and Henikoff, 2009). Depending on the size and localization of the centromere, eukaryotic chromosomes can be classified as monocentric or holocentric. The most prevalent monocentric chromosomes assemble centromeres on a single defined region, whereas holocentric ones do so along the whole length of the chromosome (nematodes, arachnids, and insects (Schvarzstein et al., 2010). Monocentric chromosomes can have two defined varieties of centromeres; the point centromere, as the one present in *Saccharomyces cerevisiae*, which is formed on a small stretch of centromeric DNA (~125 bp in budding yeast). The second type is a regional centromere that is nucleated on a larger chromosomal domain, ranging from a few kilobases in fungi such as *Schizosaccharomyces pombe* and *Candida albicans*, to hundreds of kilobases in most plants and animals (Malik and Henikoff, 2009).

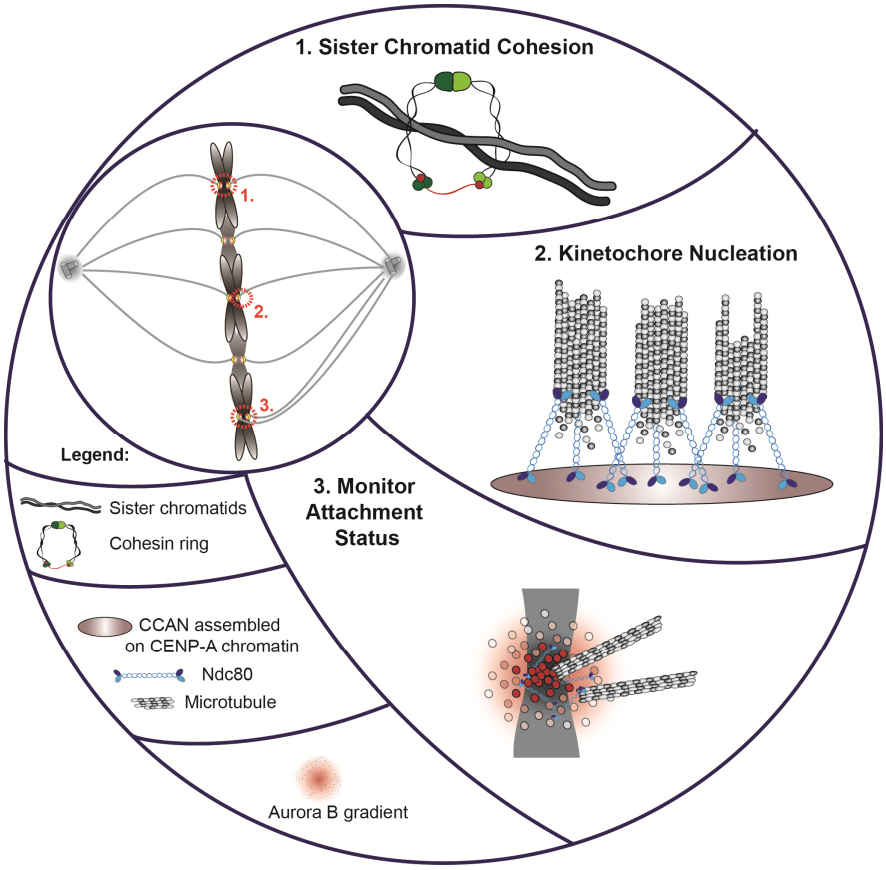

Figure 1.2 Centromeres control chromosome segregation and mitotic progression. (1) Sister chromatid cohesion is maintained at pericentric heterochromatin in mitosis to prevent premature chromosome separation (image adapted from: Mirkovic and Oliveira, 2017). (2) Centromeres form a structural platform for kinetochore nucleation, during mitosis. The latter includes the microtubule binding protein Ndc80 which allows chromosome segregation. (3) An Aurora B gradient originating from the inter-centromere region destabilizes proximal kinetochore-microtubule interactions to prevent asymmetric chromosome segregation.

1.6 Centromeric DNA

Since centromeres are directly associated with centromeric DNA, early models postulated that specific features of centromeric DNA are directing nucleation of the functional centromere. This is indeed true in the case of

the point centromere of *S. cerevisiae* that is defined by a specific DNA sequence found on all chromosomes (Clarke and Carbon, 1983). This centromeric DNA sequence is comprised of three functional elements termed centromere DNA element I (CDEI), CDEII and CDEIII. Combined they form a sequence of approximately 125 bp that is sufficient to confer mitotic stability when introduced into plasmids (Clarke and Carbon, 1980; Fitzgerald-Hayes et al., 1982; Hieter et al., 1985). The sequences of CDEI and CDEIII are conserved among all *S. cerevisiae* chromosomes, with CDEII organizing a single centromeric nucleosome containing the *S. cerevisiae* homologue of CENP-A called Cse4 (Meluh et al., 1998; Stoler et al., 1995).

Centromeres in most other organisms appear to be determined in DNA sequence independent fashion, insofar as that specific DNA sequences driving centromere assembly have not been identified. Instead of assembling on the specific DNA sequence, the majority of regional centromeres associate with highly repetitive tandem sequence repeats (Choo, 2001; Tyler-Smith and Floridia, 2000). In humans, the best characterized repeat unit is a 171 bp monomer known as α-satellite (or alphoid) DNA (Willard, 1985, 1990). These repeats exist in two distinct subtypes, type I and type II. Type I repeats, also known as α-I satellite DNA contain a 17 bp sequence termed the CENP-B box that recruits the conserved centromere protein B (CENP-B) (Earnshaw et al., 1987; Ikeno et al., 1994; Masumoto et al., 1989). α-I satellite repeats are flanked by α-II satellite DNA which contains divergent repetitive sequences and retrotransposons. Whereas repeat unit length tends to be similar between different organisms (e.g. 171 bp for primates, 186 bp in fish, 155 bp in insects) (Henikoff et al., 2001), the nucleotide sequence of these repeats displays high variability even between closely related species. Additionally,

multiple eukaryotic subfamilies display variable genomic localization of mitotically active centromeres, which shifted along the chromosome, independently of the surrounding sequences or structural rearrangements (Montefalcone et al., 1999; Rocchi et al., 2012; chapter 5 of this thesis).

One of the most compelling evidence arguing against the role of DNA sequence as principal determinant of centromere localization came with the discovery of centromeres on atypical loci. These so-called neocentromeres, initially identified in 1993 on a mitotically stable derivative of chromosome 10, lacking typical centromeric sequence as well as the CENP-B protein that binds to those sequences (Voullaire et al., 1993). Up to this date, more than 130 unique human neocentromeres, spanning all chromosomes except 22, have been identified (Liehr, 2014; Marshall et al., 2008). Neocentromere genomic location is stably maintained throughout cell divisions where they confer mitotic stability to carrier chromosomes and, in some cases, neocentromeres are inherited through human generations (Amor et al., 2004; Capozzi et al., 2009; Knegt et al., 2003; Tyler-Smith et al., 1999; Ventura et al., 2004; Wandall et al., 1998), pointing to their meiotic stability as well. Importantly, large arrays of vacated α-satellite sequences do not display any centromeric function and can be retained on neocentric chromosomes, including meiotically stable ones (Bukvic et al., 1996; Hasson et al., 2011; Liehr et al., 2010; Tyler-Smith et al., 1999). Therefore, the case of the neocentromere argues that centromeric sequences are neither necessary nor sufficient for centromere specification in human cells.

Although not strictly required for establishment of an active centromere, specific features of centromeric DNA may have a contributory role in centromere specification. One well known feature of mammalian

centromeric DNA is the recruitment of CENP-B, a sequence specific DNA binding protein that recognizes a sequence, named CENP-B box, found within a proportion of α-satellite monomers (Masumoto et al., 1989). Although the absence of CENP-B protein doesn't impair viability (Hudson et al., 1998), the presence of CENP-B boxes together with α-satellite DNA was found to be essential for formation of functional *de novo* centromere on artificial human chromosomes (Ohzeki et al., 2002). Additionally, the presence of CENP-B was found to enhance mitotic fidelity of human centromeres through stabilization of the kinetochore nucleating component of CCAN, CENP-C (Fachinetti et al., 2015; Hoffmann et al., 2016). The intricate relationship between repetitive centromeric DNA and centromere evolution is further discussed in the chapter 5 of this thesis.

1.7 CENP-A is epigenetically marking active centromeres

Epigenetic traits are heritable features whose propagation is not solely driven by underlying DNA sequences. As outlined above, centromeric DNA appears not to have a critical role in driving formation of a functional centromere. The current consensus in the centromere field is that the centromere-specific histone H3 variant CENP-A lies at the core of a positive epigenetic feedback loop and is sufficient to initiate and propagate centromeres. CENP-A, along with CENP-B and CENP-C were among the first centromere proteins to be identified using antibodies isolated from auto-immune sera from human scleroderma patients (CREST) (Earnshaw and Rothfield, 1985). These sera stained proteins at all active centromeres but, importantly, they are absent from an inactive centromere, suggesting a ´´chromatin based regulation´´ of the centromere (Earnshaw and Migeon, 1985). Soon after its initial discovery CENP-A was found to copurify with core histone proteins (Palmer et al., 1987) and have histone-like properties

(Sullivan et al., 1994). In addition, centromere specific CENP-A homologues exist in nearly all species analyzed so far (Malik and Henikoff, 2003; Talbert et al., 2012), with the exception of kinetoplastids and some holocentric insects that do not appear to contain a recognizable CENP-A homologue (Akiyoshi and Gull, 2013; Drinnenberg et al., 2014). A remarkable feature of centromeric chromatin is its requirement for the maintenance of centromeric chromatin across the germline in several, but not all organisms analyzed thus far. In mammals, early work has shown that CENP-A is present in mature bovine sperm, evading protamine deposition (Palmer et al., 1990), suggesting CENP-A may play a transgenerational role in mammals. Indeed, stable paternal transmissions of neocentormeres within human families demonstrate that the position of the centromere is inherited epigenetically at least through the male germline (Amor et al., 2004; Tyler-Smith et al., 1999). Sperm retained CENP-A was also found in *X.laevis* and *D. melanogaster* (Dunleavy et al., 2012; Milks et al., 2009; (Dunleavy et al., 2012; Milks et al., 2009; Raychaudhuri et al., 2012). In Drosophila, a causative role for CENP-A in germline centromere maintenance has been shown. Selective removal of the CENP-A homologue [known as CID or cenH3 (Talbert and Henikoff, 2013)] from paternal centromeres resulted in successful fertilization but in the selective failure to segregate paternal chromosomes in the zygote, despite normal segregation of maternal chromosomes and the availability of a maternal pool of CID (Raychaudhuri et al., 2012). The transgenerational necessity of CENP-A is not universal in life. *C. elegans*, sperm is devoid of CENP-A which is provided de novo through the maternally deposited pool of CENP-A (Gassmann et al., 2012). Further, during oogenesis, pre-existing CENP-A is removed, and is de novo deposited (Monen et al., 2005).

In proliferating somatic cells, loss of CENP-A is lethal due to the severe defects in chromosome segregation in all species analyzed (Black et al., 2007; Blower and Karpen, 2001; Buchwitz et al., 1999; Fachinetti et al., 2013; Henikoff et al., 2000; Howman et al., 2000; Régnier et al., 2005; Stoler et al., 1995; Talbert et al., 2002). Additionally, CENP-A is sufficient for the recruitment of virtually all known centromere and kinetochore proteins (Barnhart et al., 2011; Carroll et al., 2009; Foltz et al., 2006; Guse et al., 2011; Heun et al., 2006; Liu et al., 2006; Mendiburo et al., 2011; Okada et al., 2006), with the exception of the sequence specific DNA binding protein CENP-B (Pluta et al., 1992; Voullaire et al., 1993). In a groundbreaking study, (Mendiburo et al., 2011) used Drosophila S2 cells to tether CENP-A to a naïve chromatin domain containing Lac operator sequences (using a LacI DNA binding domain), not previously associated with centromere function. Once tethered, CENP-A^{CID}-LacI creates a local nucleosome pool that is able to recruit virtually all known downstream centromere and kinetochore proteins allowing stable binding of microtubules. Importantly, once formed, this nascent centromere recruited naïve CENP-A^{CID}, not previously associated with this region, even after the initial tether had been lost, indicative of self-propagation of CENP-A^{CID}. Analogous experiments were performed with the CENP-A loading factor HJURP. In this case, not only *de novo* centromere formation was observed (Barnhart et al., 2011; Hori et al., 2013) but this centromere was shown to rescue chromosome stability and cell viability after deletion of the endogenous centromere in chicken DT40 cells (Hori et al., 2013). Exploiting conditional endogenous centromere deletion combined with artificial genomic targeting of LacI-fused CCAN components to LacO array, (Hori et al., 2013) demonstrated that CENP-C and CENP-I are sufficient to initiate a heritable centromere at the LacO array, as observed in the case of LacI-

HJURP tethering. This ectopic centromere functionally replaced the endogenous one and faithfully maintained chromosome Z ploidy in the cell population. Although these two CCAN components have been shown to be required for CENP-A assembly at endogenous centromeres (Okada et al., 2006; Erhardt et al., 2008; Carroll et al., 2010), these results show they can also be sufficient for *de novo* recruitment of CENP-A on naive chromatin. Importantly, this implies that although CENP-A chromatin provides a stable heritable core, its propagation involves a positive epigenetic feedback mechanism in which other CCAN components, themselves dependent on CENP-A, play an active role in CENP-A recruitment.

1.8 CENP-A nucleosomes are stably propagated at centromeres through mitotic and meiotic divisions

Early work indicates that total cellular CENP-A protein exhibits a remarkably long half-life and lives as long as the cell itself, equating ~50% decrease per cell generation (Shelby et al., 1997). The apparent slow turnover required the employment of specific tools to assess protein dynamics. Fluorescence recovery after photobleaching (FRAP) which relies on local, irreversible photo-bleaching of a fluorophore, followed by subsequent repopulation of a bleached area with unbleached molecules provides information of the local rate of protein turnover. FRAP experiments on budding yeast kinetochores (containing a single microtubule attachment site), revealed that the yeast CENP-A homologue, Cse4 displays very low turnover rates at centromeres except during S phase where all of the preexisting Cse4 nucleosomes are exchanged (Pearson et al., 2004). Cse4 was found to be stable specifically at the centromere, whereas the non-centromeric Cse4 is degraded via ubiquitin-mediated proteolysis (Collins et al., 2004). Stable binding of Cse4 at centromeres was recently confirmed in

elegant experiments using a photoconvertible Cse4-tdEos (Wisniewski et al., 2014). Eos, green in the unconverted state can be stably switched to red emission upon short wavelength excitation. Following conversion, Cse4 molecules were found to be stably associated with centromeres until their turnover during DNA replication.

Stability of the fission yeast, kinetochore-bound, CENP-A homologue was demonstrated using photobleaching of Cnp1-GFP (Coffman et al., 2011), which displayed a similar dynamics as previously described for Cse4 (Pearson et al., 2004). Interestingly, in contrast to the yeasts, holocentric *C. elegans* embryos, characterized by extremely short division times (~15min), photobleaching of embryonic CeCENP-A-GFP in anaphase in the one-cell embryo results in the complete fluorescence recovery in the next cell division, indicative of complete loss of pre-existing CeCENP-A nucleosomes (Gassmann et al., 2012). Here, sites for CeCENP-A deposition appear to be based on other genomic features rather than pre-existing CENP-A. These regions include those with low transcriptional activity in the parental germline (Gassmann et al., 2012) and sites of high DNA accessibility (Steiner and Henikoff, 2014).

In vertebrate cells, following the initial determination of CENP-A stability with a tagged but overexpressed shut-off allele in human cells (Shelby et al., 1997), a shut-off in the context of a full deletion of the CENP-A gene in chicken DT40 cells (Régnier et al., 2005) revealed that the loss rate of the cellular CENP-A pool is very slow indeed, with the first mitotic defects occurring only after 7-8 cell cycles. Similar results were obtained in human cells after conditional deletion of CENP-A (Fachinetti et al., 2013). The fact that these cells can survive for extended amount of time without continuous supply of fresh CENP-A, strongly suggests that pre-existing CENP-A, once

assembled into nucleosomes, remains stably bound to centromeric chromatin. While these studies determined that CENP-A turns over slowly, establishing the actual turnover rate proved difficult to determine. The FRAP methodology is suitable for determining protein dynamics at short time scales such as in organisms which have a short cell division time, but proofs limited for dissecting protein turnover and replenishment rates at long time intervals. This limitation was surmounted by the use of a fluorescent pulse labeling strategy such as SNAP-tag technology, which allows for pulse labeling and visualization of different cohorts of the same protein within whole cell populations. SNAP is a derivative of a human DNA repair enzyme, O6-alkylguanine-DNA alkyltransferase (AGT). The endogenous AGT enzyme recognizes O6-alkylated guanine in DNA, and transfers the alkyl group to a reactive cysteine residue. This self-labeling capacity is exploited in a mutant version of AGT (named SNAP) which has a high affinity towards synthetically engineered small, cell permeable molecules, such as benzylguanine (BG) (Keppler et al., 2003). The enzymatic reaction between SNAP and its substrate is irreversible, highly efficient and specific. Combining serial labeling of SNAP-tagged proteins with different SNAP substrates enables visualization and fate determination of pre-existing versus newly synthesized pools of the same protein (Bodor et al., 2012). Following a pulse labeled cohort of CENP-A-SNAP molecules over the course of 48-72 hours, demonstrated the stable transmission of CENP-A through mitotic divisions (Bodor et al., 2013; Jansen et al., 2007). The loss rate of this pool was found to equate ~50% during each cell division, consistent with quantitative recycling of old CENP-A during S phase, with no additional turnover (Bodor et al., 2013; Dunleavy et al., 2011; Jansen et al., 2007). This high rate of retention appears to be unique to CENP-A nucleosomes. Similar pulse labeling experiments on H3.1 and

H3.3 did not reveal such retention at centromeric chromatin (Bodor et al., 2013; Falk et al., 2016), indicating that the property of stable transmission is linked to CENP-A itself, not the centromeric chromatin environment as a whole. However, histone H4 shows a striking differential stability. In the genome overall its turnover rates are similar to that of H3.1, but at the centromere H4 is retained to the extent of CENP-A (Bodor et al., 2013). CENP-A directly contacts H4 in the prenucleosomal complex as well as within the nucleosome, forming a highly rigid structure (Black et al., 2004, 2007), likely directly stabilizing H4 at the centromere. The other remaining nucleosome partners, H2A and H2B, like H3.1 and H3.3 do not display any elevated retention at the centromere (Bodor et al., 2013). Hence, CENP-A/H4 forms a stable subnucleosomal complex that represents the epigenetic core of the centromere which is quantitatively maintained throughout multiple cell divisions.

The most striking example showcasing extreme stability of CENP-A nucleosomes is recent work in female mouse meiosis (Smoak et al., 2016). Like in humans, mouse oocytes are arrested in meiotic prophase I for an extended period of time. CENP-A is readily detected in arrested mouse oocytes. However, no assembly occurs at any appreciable rate. Consistent with that observation, deletion of the CENP-A in early oogenesis has no impact on long term (~1 yr) retention of centromeric CENP-A despite the lack of a nascent pool.

1.9 Determinants of CENP-A stability

The molecular underpinnings enabling the remarkable CENP-stability are, at least in part, embedded within CENP-A nucleosome itself. H3 and its isoform CENP-A, share substantial degree of common features, including ~75% sequence similarity in their histone fold domain (HFD). On the other

hand, a very low level of homology exists between the N-terminal histone tails of H3 and CENP-A, suggesting that this could be a differential feature functionally separating these two histones (Palmer et al., 1991; Sullivan et al., 1994). Unexpectedly, using chimeric proteins of H3 and CENP-A, it was shown that the HFD rather than the tail of CENP-A is responsible for its centromere targeting (Sullivan et al., 1994). The portion of CENP-A that confers its centromere targeting lies within this domain (HFD), in a subdomain termed CENP-A targeting domain (CATD), consisting of loop1 and the α2-helix (Black et al., 2004) (Figure 1.3A). Replacement of the equivalent domain in H3 with that of CENP-A is sufficient to target an $H3^{CATD}$ chimera to centromeres (Black et al., 2004, 2007) and neocentromeres (Bassett et al., 2010). Importantly, the CATD confers increased conformational rigidity to (CENP-A/H4)2 tetramers as well as CENP-A nucleosomes (Black et al., 2004). In addition, the CATD is directly recognized by its specific chaperone and assembly factor, HJURP (albeit different residues participate in HJURP recognition from those that are responsible for increased rigidity) (Bassett et al., 2012) (Figure 1.3B), which targets and deposits nascent CENP-A to centromeres (Dunleavy et al., 2009; Foltz et al., 2009; Shuaib et al., 2010). Remarkably, complete genetic substitution of endogenous CENP-A with $H3^{CATD}$ showed that this chimera retained the capacity to maintain its own centromeric levels over multiple cell cycles, suggesting that CATD is the critical subdomain responsible for longevity of CENP-A nucleosome *in vivo* (Fachinetti et al., 2013). Importantly, $H3^{CATD}$ chimeras display identical loading dynamics as wild type CENP-A, and crucially, induce increased stability of a direct binding partner of CENP-A-histone H4 (Bodor et al., 2013).

Taken together, these results indicate that long-term stability of CENP-A nucleosomes is conferred by its CATD, which allows for centromeric

maintenance of not only CENP-A, but $(CENP\text{-}A/H4)_2$ subnucleosome core as well. Interestingly, a C terminal LEEGLG motif of CENP-A (absent from H3), is responsible for the recruitment of the majority of downstream centromere and kinetochore proteins (Carroll et al., 2010; Fachinetti et al., 2013; Guse et al., 2011). This appears to be the main reason why a $H3^{CATD}$ chimera could, for a limited amount of time, reside at the centromere but was not sufficient to rescue a complete depletion of endogenous CENP-A. Thus, the CATD seems to be mostly dispensable for CENP-A function (recruitment of kinetochore proteins); rather it is responsible for maintenance of centromere identity.

In summary, the CATD emerges as a differential feature of CENP-A nucleosomes functionally separating CENP-A and H3 histones and implies that the extreme stability of CENP-A nucleosomes is encoded within CENP-A molecule itself. Recent work however defined CENP-C, a member of CCAN network, as an additional extrinsic factor contributing to CENP-A stability. CENP-C binds directly to chromatin-bound CENP-A, and as a consequence, induces structural changes in conformation of CENP-A nucleosomes. This results in increased rigidity of CENP-A nucleosomes, a feature likely contributing to its stable maintenance at centromeres, since CENP-C depletion causes a rapid loss of CENP-A from the chromatin (Falk et al., 2015).

1.10 The Constitutive Centromere-Associated Network (CCAN)

CENP-A acts as the most upstream component in kinetochore assembly by specifying the point of contact between the DNA and mitotic spindle. CENP-A directs the formation of the constitutive centromere associated network (CCAN) which in turn, during mitosis, recruits a secondary protein complex known as the kinetochore (Fukagawa and Earnshaw, 2014). The

constitutive centromere-associated network (CCAN) is composed of 16 proteins (Amano et al., 2009; Foltz et al., 2006; Hori et al., 2008; Obuse et al., 2004; Perpelescu and Fukagawa, 2011) among which only two directly recognize chromatin-incorporated CENP- molecules: CENP-C and CENP-N. CENP-C and CENP-N recognize distinct domains in the CENP-A nucleosome; CENP-N recognizes the CATD domain embedded within the HFD (Figure 1.3C) of CENP-A whereas CENP-C recognizes the C-terminal LEEGLG motif of CENP-A (Carroll et al., 2009, 2010; Logsdon et al., 2015). Recently, it has been shown that both the CATD and the N-terminal portion of CENP-A, together with its C terminal tail are contributing to the efficient recruitment of CENP-C to an ectopic genomic locus (LacO array in this case) (Logsdon et al., 2015) (Figure 1.3D)

CENP-N is required for mitotic progression and accurate chromosome segregation (Carroll et al., 2009; Foltz et al., 2006; McClelland et al., 2007). Centromeric localization of CENP-N is necessary for centromere localization of several CCAN components, including CENP-H, CENP-I, CENP-K, CENP-C and CENP-O (Carroll et al., 2009; McClelland et al., 2007). However, CENP-N doesn't appear to be involved in recruitment of kinetochore proteins since depletion of CENP-N doesn't affect the levels of the Nnf1 component of the Mis12 complex (McClelland et al., 2007). CENP-N and CENP-A centromere presence appear to be mutually dependent since depletion of CENP-N causes defective loading of nascent CENP-A (Carroll et al., 2009), and rapid destruction of CENP-A using an auxin-degron system leads to diminished centromere deposition of new CENP-N molecules in S phase (Hoffmann et al., 2016).

CENP-C is a DNA binding protein that localizes to inner centromere in a CENP-A dependent manner (Saitoh et al., 1992). CENP-C homologues

have been identified in virtually all model organisms, including yeast, flies, plants and mammals, and have been shown to be required for proper chromosome segregation and mitotic progression (Dawe et al., 1999; Erhardt et al., 2008; Fukagawa et al., 2001; Moore and Roth, 2001; Oegema et al., 2001; Schuh et al., 2007; Tomkiel et al., 1994). Interestingly, CENP-C is the only CCAN component thus far identified in Drosophila, which may explain why centromeric localization of CENP-A^{CID} is absolutely dependent on CENP-C (Erhardt et al., 2008). CENP-C is required for the centromere localization of several kinetochore proteins, including Knl1, the Mis12 complex and the Ndc80 complex that together are known as the KMN network, which forms the principal microtubule binding complex in the kinetochore (Cheeseman et al., 2006; Klare et al., 2015; Milks et al., 2009; Screpanti et al., 2011). CENP-C is also required for the recruitment of checkpoint proteins, for mitotic checkpoint function (Kwon et al., 2007; Przewloka et al., 2011; Screpanti et al., 2011), and for the centromere localization of other CCAN components such as CENP-H, CENP-I, CENP-K and CENP-T (Carroll et al., 2010). In humans, CENP-C deposition occurs a few hours after CENP-A assembly, and the presence of CENP-A nucleosomes is absolutely required for centromeric targeting of CENP-C (Hoffmann et al., 2016). In accordance to direct binding of CENP-C to CENP-A nucleosomes, excision of endogenous CENP-A, its C terminal domain or acute degradation of CENP-A using and auxin-degron system, leads to the proportional reduction in the amount of centromeric CENP-C (Fachinetti et al., 2013; Hoffmann et al., 2016). However, after the initial reduction, the levels of CENP-C become stabilized even when total amount of CENP-A is reduced to ~1% of its initial level. This retention of CENP-C at the centromeres is enhanced by the DNA binding CENP-B protein that recognizes the amino tail of CENP-A nucleosomes and

adjacent alphoid DNA (Figure 1.3E); once at the centromeres, CENP-B further stabilizes the levels of centromeric CENP-C through a direct protein-protein interaction, thereby providing an additional parallel pathway for CENP-C dependent kinetochore assembly (Fachinetti et al., 2015; Hoffmann et al., 2016) (Figure 1.4). Therefore, at centromeres, CENP-C has a dual role: on one hand it stabilizes centromeric CENP-A nucleosomes, thus contributing to its maintenance across cell cycle; and on the other, forms a critical link between inner centromere domain and the kinetochore proteins that bind plus ends of spindle microtubule.

Another member of the CCAN is CENP-T, which together with its binding partners CENP-S, CENP-W and CENP-X forms a platform for binding of proteins that in turn interact with microtubules (Amano et al., 2009; Foltz et al., 2006; Hori et al., 2008, 2013; Obuse et al., 2004) (Figure 1.4). CENP-T and -W form a hetero-tetramer with CENP-S and –X and shows DNA binding activity. It protects a ~100 bp region of nucleosome-free DNA forming a nucleosome-like structure (Nishino et al., 2012). The CENP-T-W complex does not directly associate with CENP-A, but with histone H3 in the centromere region (Hori et al., 2008). Consistently, deposition of nascent CENP-T does not solely depend on CENP-A nucleosomes (Nishino et al., 2012). However, recently it has been shown that both N and C-terminal tails of CENP-A, together with CATD promote recruitment of CENP-T to ectopic centromeres (Logsdon et al., 2015). Whether this CENP-A driven recruitment of CENP-T resulted in the formation of CENP-T/W/S/X complex is not known. However, it is likely that another adaptor protein (possibly a member of CCAN) is mediating the interaction between CENP-A and CENP-T, given that CENP-T remains mostly unaffected by rapid CENP-A degradation, which is a sharp contrast to the members of

CCAN that are directly interaction with CENP-A nucleosomes (Hoffmann et al., 2016).

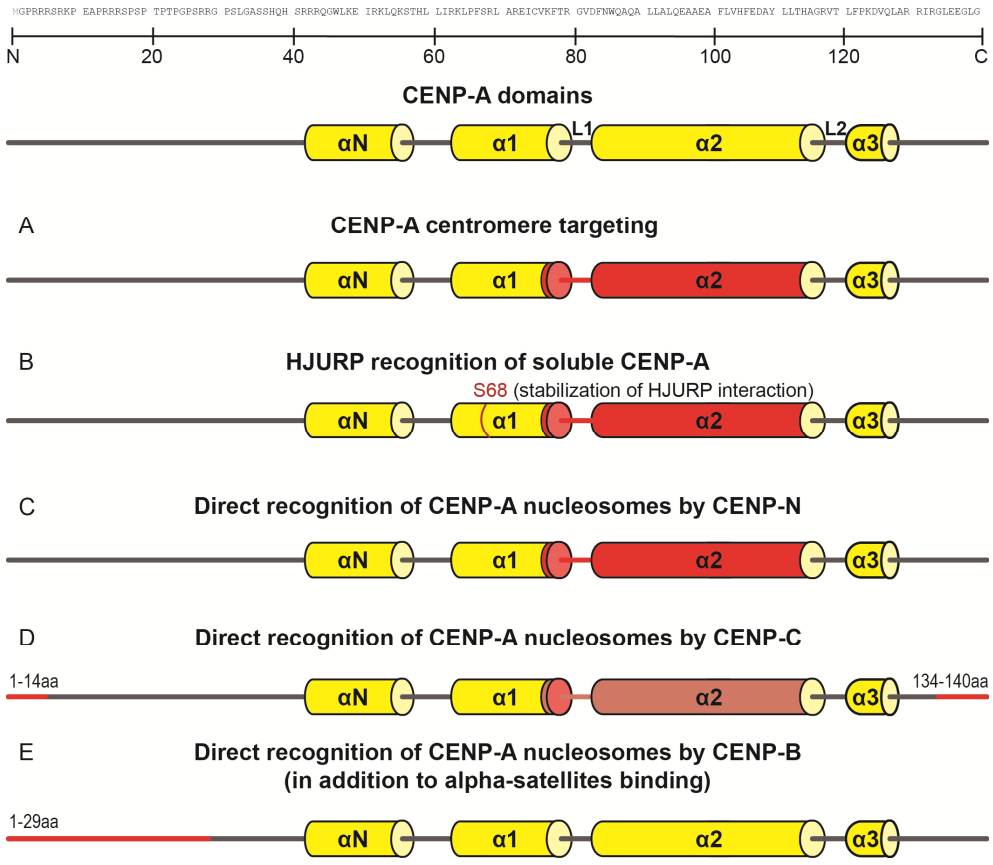

Figure 1.3 CENP-A protein sequence along with all relevant domains [alpha N helix (αN), alpha-1 helix (α1), Loop-1 (L1), alpha-2 helix (α2), Loop-2 (L2), alpha-3 helix (α3)] are depicted (yellow). Domains that are responsible for specific features of CENP-A are highlighted in red. (A) Centromere targeting domain (CATD) is allowing differentiation between CENP-A and H3, and is responsible for centromeric targeting of CENP-A. (B) HJURP recognizes CATD within CENP-A/H4/HJURP prenucleosomal complex. Serine 68 located outside of CATD further stabilizes this interaction. (C) CENP-N directly recognizes CATD in CENP-A nucleosomes. (D) CENP-C directly recognizes the C-terminal LEEGLG sequence of CENP-A nucleosomes. Additionally, CENP-C recognizes N terminal portion of

Figure 1.3 Continued: CENP-A nucleosomes, where it interacts with CENP-B. (E) CENP-B binds to CENP-B boxes in alpha satellites and recognizes N terminal domain of CENP-A.

The N terminus of CENP-T proteins interacts directly with the Ndc80 complex in the outer kinetochore (Gascoigne et al., 2011; Nishino et al., 2013). The CENP-T/-W complex is not maintained at centromeres through cell divisions, and new deposition is absolutely required at each cell cycle for kinetochore function. Consistently, CENP-T and CENP-W are loaded and become enriched at centromeres during late S and G2 phases, just before the recruitment of the KMN network to the kinetochores (Prendergast et al., 2011).

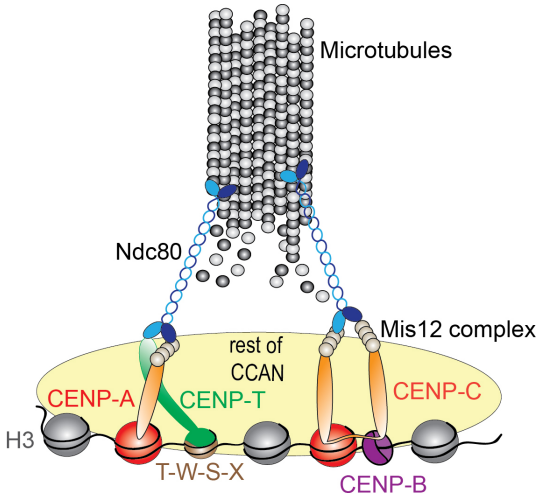

Figure 1.4 The CCAN connects centromeric DNA and microtubule plus ends. CENP-T/W/S/X complex forms a bridge between centromeric chromatin and the mitotic kinetochore through a direct interaction between CENP-T and the Ndc80 microtubule-binding complex. CENP-C (which directly recognizes CENP-A nucleosomes) binds the remaining of CCAN and the Mis12 complex, which in turns directly interacts with Ndc80 complex. CENP-C forms a dimer and interacts with the DNA sequence specific protein CENP-B. CENP-B functions as a dimer and binds CENP-B boxes at alpha satellites. Simultaneously, CENP-B directly

Figure 1.4 Continued: interacts with CENP-C, resulting in increased mitotic fidelity of a CENP-C dependent branch of kinetochore nucleation.

Remarkably, CENP-A *per se* is expendable for proper mitotic division given that acute destruction of CENP-A (employing an auxin-degron system), 4h prior to mitosis doesn't significantly influence proper chromosomes segregation (Hoffmann et al., 2016). However if CENP-A is depleted prior to critical time window when CCAN components are targeted to the centromere, the integrity of whole kinetochore is diminished, thus leading to higher frequency of mitotic errors. Taken together, these results indicate that CENP-A containing nucleosomes provide the initial structural platform for the assembly of the kinetochore. Once assembled, building blocks of kinetochore are autonomous in regard to CENP-A, as exemplified by the stable microtubule-kinetochore attachment during mitosis and faithful genome segregation occurring in the absence of CENP-A.

1.11 The modularity of CENP-A dependent kinetochore assembly

As outlined above, CENP-A nucleosomes actively participate in the nucleation of kinetochore, while being dispensable for its maintenance after the initial establishment. Current models for centromere and kinetochore architecture are based on repeated individual subunits, in which the amount of centromere components directly dictates the number of downstream kinetochore proteins, and ultimately the number of microtubule attachment sites. This form of organization was initially proposed in 1991, when islets of proteins recognized via CREST antibodies were identified in a stretched centromeric DNA fiber (Zinkowski et al., 1991). Evidence for such a modular organization is found at the *S. cerevisiae* point centromere in which the proteins forming the interface between centromeric chromatin and the microtubule plus end exist in specific stoichiometries (Joglekar et

al., 2006). Regional centromeres tend to assemble on large stretches of centromeric DNA compared to the budding yeast point centromeres and they are bound by multiple spindle microtubules [ranging from 2-4 in fission yeast to ~17 in the case of humans (McEwen et al., 2001; Sagolla et al., 2003). Initial studies, focused on the centromeres of fission yeast and *C. albicans* (Joglekar et al., 2008), found a strikingly constant ratio between the amount of centromeric CENP-A nucleosomes, structural components of kinetochore and number of microtubules attached during mitosis. In these cases, while absolute numbers differ, the number of kinetochore proteins per microtubule attachment are very similar between budding and fission yeast. For both yeasts there are 6-8 molecules of KMN network per kinetochore-microtubule attachment. These findings strongly argue that the regional centromeres of fission yeast are composed of repeated structures reminiscent of the ones existing in budding yeast. This apparent kinetochore architecture extends to certain metazoan species, such as chicken DT40 cells, in which the copy number of CCAN network members (namely CENP-C, CENP-H, CENP-I and CENP-T) is in nearly stoichiometric relation to KMN network members (Mis12, Knl1 and Ndc80), which, once again, assemble at ~8 molecules per microtubule (Johnston et al., 2010).

However, a direct relationship between the number of centromeric CENP-A nucleosomes and amount of downstream kinetochore components is incompatible with the fact that constitutive overexpression of Cnp1 does not lead to significant changes in the copy number of kinetochore protein (Joglekar et al., 2008). Consistently, in *C. albicans*, the number of CaCse4 nucleosomes is larger than the number of microtubule attachment sites (Joglekar et al., 2008), indicating that the relationship between centromeric chromatin and microtubule attachment sites is less defined. This notion is

further supported by the fact that CENP-A depletion in human cells resulting in ~7% of total centromeric (Fachinetti et al., 2013) or ~10% of cellular pool (Liu et al., 2006) had no effect on centromere integrity at least in the short term. Upon partial loss of CENP-A, proteins such as CENP-C and CENP-T remain largely unaffected (Fachinetti et al., 2013). In an extreme case, upon complete acute complete loss of CENP-A, the centromere remains mitotically functional at least initially, after which failure to propagate the centromere in the next division results in gradual loss of centromere components (Hoffmann et al., 2016). In agreement with the stoichiometric disconnect between centromeric chromatin and the rest of the centromere, altering CENP-A levels in human RPE cell line between 40% and 240% relative to wild type, showed no significant effect on the amount of critical kinetochore proteins (Bodor et al., 2014). These included CENP-C and CENP-T, which are responsible for mitotic recruitment of the KMN network (Gascoigne et al., 2011), as well as the key microtubule binding protein Hec1/NDC80 (Cheeseman et al., 2006; DeLuca et al., 2006). Taken together, these results argue that on a typical human centromere the amount of CENP-A nucleosomes is in excess compared to the critical number necessary to maintain the centromere, which could in part be facilitated through semi-stable self-regulated recruitment of downstream CCAN proteins.

Another insight into the relationship between CENP-A chromatin and the kinetochore comes from overexpression studies. Excess CENP-A results in its mislocalization to non-centromeric sites (Athwal et al., 2015; Heun et al., 2006; Lacoste et al., 2014). Mistargeted CENP-A is not randomly distributed; rather it is enriched at sites of high histone turnover (Athwal et al., 2015; Lacoste et al., 2014). Even at physiological expression levels, CENP-A is present outside the centromere in a surprisingly high amount

(Bodor et al., 2014). Quantitative fluorescence microscopy methods have estimated that only ~20% of CENP-A is centromeric and about half of all CENP-A is chromatin bound elsewhere (Bodor et al., 2014)(Figure 1.5). However, due to the large genome size these CENP-A nucleosomes represent less than one in a thousand nucleosomes, compared to ~50 fold higher enrichment at centromeres (Bodor et al., 2014). In human retinal pigment epithelium (RPE) cells there are ~400 molecules per centromere (Bodor et al., 2014). Given the predominantly octameric nature of CENP-A nucleosomes (Black and Cleveland, 2011; Hasson et al., 2013), this number converts into ~200 CENP-A nucleosomes in interphase, which are split into ~100 nucleosomes on mitotic centromeres. This number is low compared to the size of chromatin at the centromere. The scarcity of CENP-A nucleosomes at the centromere (1 in 25 compared to H3 on average) (Bodor et al., 2014) appears to be inconsistent with the stable maintenance of a self-templating positive feedback loop, which typically relies on local cooperativity (Dodd et al., 2007). However, analysis of nucleosome distribution at neocentromeres, where such analysis is possible, shows that CENP-A nucleosomes tend to be organized in clusters, as also found by chromatin fiber analysis (Blower et al., 2002). Within these clusters, individual positions harbor CENP-A with a remarkably high occupancy (up to 80% of total cells (Bodor et al., 2014)), indicative of sites with a strong nucleosome positioning activity favoring CENP-A. Therefore, strong enrichment of CENP-A nucleosomes coupled with their possible clustering at the centromere likely provides an ample amount of CENP-A nucleosomes sufficient to maintain a positive epigenetic feedback loop on one hand and form a platform for kinetochore nucleation during mitosis on the other (Figure 1.5). Therefore, it is possible to envision a scenario in which these sporadic non-centromeric CENP-A nucleosomes

might have a limited capacity to attract some centromeric components, particularly those that directly interact with CENP-A (Gascoigne et al., 2011). However, if the local pool of CENP-A does not reach a critical threshold, such centromere protein recruitment is insufficient to initiate the formation of a functional centromere (Figure 1.5). Therefore, rather than maintaining a linear relationship between CENP-A nucleosomes and downstream components, the CCAN and the kinetochore, once formed, maintain an internal stoichiometry and become to some extend independent of fluctuation in the centromeric CENP-A pool size.

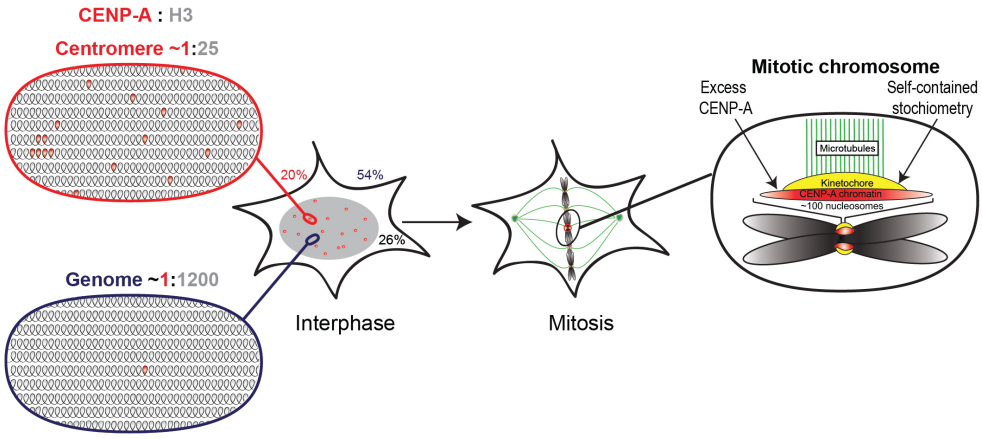

Figure 1.5 An integrated view of human centromere architecture. Left: Interphase distribution of CENP-A relative to histone H3 at an average human centromere (top) and whole genome level (bottom) adapted from (Bodor et al., 2014). Right: Organization of mitotic chromosome in which individual centromeres contain ~100 CENP-A nucleosomes, which is in excess of what is required to nucleate the kinetochore of a fixed size.

One curious case in which the levels of centromeric CENP-A appear to dictate the amount of downstream kinetochore proteins has been reported to occur during meiosis in mice (Chmátal et al., 2014). In mammals, during female oogenesis only one out of four meiotic product will give rise to the future gamete. The probability for any allele to be transmitted should, in

principle, follow Mendelian rules of inheritance. However, certain ´´selfish´´ genomic elements can skew this ratio and are preferentially retained in the mature egg, a process known as meiotic drive. The (Chmátal et al., 2014) study showed that the amount of kinetochore proteins assembled at the meiotic centromere correlates with the amount of CENP-A nucleosomes. Chromosomes having fewer CENP-A nucleosomes at the centromere relative to the other ones, assembled a lower amount of Hec1/NDC80, which results in its positioning near the cell cortex due to asymmetric microtubule forces within the meiotic spindle, resulting in its preferential exclusion to the polar body. The inverse was found for chromosomes with a higher amount of centromeric CENP-A nucleosomes, which were preferentially retained in the mature egg. While the resulting drive is not large, only by 10% form random (Chmátal et al., 2014), at evolutionary time-scales, this would have a profound effect on the frequency of a specific chromosome within a population. While in mitosis such inequalities maybe equalized by the mitotic checkpoint, this is much weaker during meiosis allowing for centromere discrepancies to evolve.

1.12 Propagation of centromeric chromatin across cell divisions

CENP-A nucleosomes are stably maintained and propagated at mitotic and meiotic centromeres (Bodor et al., 2013; Jansen et al., 2007; Smoak et al., 2016). This unusually slow turnover of CENP-A at each centromere (Falk et al., 2015a) has consequences for how the correct levels are maintained across subsequent cell division cycles. New CENP-A histones can either be incorporated at a continuous slow rate to compensate for the two fold reduction during S phase, or alternatively, assembly is restricted to a discrete cell cycle window to control the rate and quantity of assembly. It turns out that, in all species examined thus far, control of CENP-A

assembly is maintained by rendering it tightly cell cycle restricted rather than allowing continuous slow assembly. Given the key role of centromeres in mitosis and the fact that CENP-A is lost by two-fold during the preceding S phase, it was initially expected that the replenishment of the S phase diluted pool of CENP-A would occur prior to mitosis (Csink and Henikoff, 1998; Shelby et al., 2000). In budding yeast, CENP-A turns over during S phase (Pearson et al., 2004; Wisniewski et al., 2014). Such turnover appears to be a common feature among unicellular eukaryotes. In an interesting case of the unicellular red algae *Cyanidioschyzon merolae*, CENP-A^{CENH3} is detected at the centromeres only between S phase and mitosis, and remains undetectable in G1 phase, indicating eviction of CENP-A^{CENH3} (Kanesaki et al., 2015; Maruyama et al., 2007). Upon re-entry into subsequent S phase, CENP-A^{CENH3} is *de-novo* deposited at regional centromeres of *C. merolae* (Kanesaki et al., 2015). With the exception of these single celled organisms, CENP-A assembly appears to be uncoupled from DNA replication in metazoans and plants.

In most animal systems examined, a unique pattern of cell cycle-coupled CENP-A replenishment was uncovered where assembly of newly synthesized CENP-A is delayed until mitotic exit, in G1 phase of the next cell cycle, after the primary function of the centromere has been fulfilled. This paradoxical timing of centromeric chromatin assembly was initially discovered in Drosophila and human cells based on steady state fluorescence, FRAP experiments and SNAP-based pulse labelling, respectively (Jansen et al., 2007; Schuh et al., 2007). The SNAP technology has proven extremely useful in dissecting chromatin dynamics (Bergmann et al., 2011; Jansen et al., 2007; Prendergast et al., 2011; Ray-Gallet et al., 2011). To assay for the assembly of nascent CENP-A-SNAP specifically, the pre-existing (chromatin bound) pool of CENP-A-SNAP is

labelled with a non-fluorescent SNAP substrate (quench). During the ensuing chase period new, unlabeled CENP-A is synthesized which can be fluorescently labeled at a later time point (Bodor et al., 2012b). This methodology allows for the visualization of centromeres decorated with nascent CENP-A. G1 restricted assembly of CENP-A in human cells was confirmed by photo-bleaching experiments of CENP-A-GFP (Hemmerich et al., 2008), and later also found be the conserved in chicken DT40 cells (Silva et al., 2012b), and Xenopus (Bernad et al., 2011; Westhorpe et al., 2015).

A key question that follows is to determine how CENP-A assembly is coupled to the cell cycle to maintain correct centromere levels. Early work showed that microtubule attachment and checkpoint signaling, two key aspects of mitosis, are not required for subsequent assembly (Jansen et al., 2007; Schuh et al., 2007). Instead, mitotic passage is primarily needed to result in APC-mediated cyclin destruction and concomitant loss of Cdk activity. This notion resulted from experiments demonstrating that selective inhibition of both Cdk1 and Cdk2 (Cdk1/2) in S or G2 phase is sufficient to induce premature, premitotic CENP-A assembly (Silva et al., 2012b). CENP-A assembly commences rapidly upon Cdk inactivation, either naturally or artificially. This has led to a model in which all factors necessary for CENP-A loading are present and poised for activity prior to mitotic exit, but are held inactive due to the Cdk1/2 activities in S, G2 and mitosis, when these kinases are active. While CENP-A is the prime candidate regulating propagation of centromeric chromatin, the fact that $H3^{CATD}$ chimera still retained G1 restricted timing of loading to the centromeres argues that external binding factors are likely contributors to cell cycle dependent CENP-A assembly, compared to CENP-A itself (Bodor et al., 2013). Indeed, the CENP-A specific chaperone HJURP is exclusively

targeted to G1 centromeres (Dunleavy et al., 2009; Foltz et al., 2009) concurrent with its dephosphorylation on Cdk consensus residues as shown by (Müller et al., 2014) and discussed in chapter 3 of this thesis. Mutation of Cdk responsive residues within HJURP prior to mitotic exit is sufficient to induce limited precocious loading of CENP-A at S and G2 centromeres (Müller et al., 2014); and chapter 3 of this thesis). In addition, ectopic targeting of HJURP to centromeres prior to mitotic exit also leads to premature incorporation of CENP-A molecules, suggesting that rather than controlling the interaction interface between CENP-A and HJURP, the negative regulation occurs primarily at the level of localization of the assembly factor (chapter 3 of this thesis). Similarly, Cdk1/2 activities also negatively regulate centromeric localization of another CENP-A assembly factor, the M18 complex. This complex is targeted to centromeres in anaphase of mitosis, prior to the onset of CENP-A deposition, and its activity is necessary for subsequent steps in CENP-A deposition which involves the targeting of HJURP to the centromeres (Barnhart et al., 2011; Fujita et al., 2007). The largest member of the M18 complex, M18BP1 is under Cdk1/2 control, which limits its centromeric recruitment until loss of Cdk1 activity in anaphase (McKinley and Cheeseman, 2014; Silva et al., 2012; and Chapter 2 of this thesis). Interestingly, like HJURP, forced premature recruitment of M18BP1 to the centromeres can overcome negative cell cycle regulation to some extent (McKinley and Cheeseman, 2014; chapter 2 of this thesis). Additionally, in chapter 2 of this thesis I describe the identification of a single phosphorylation site at Threonine 653 to be the key to this control. Finally, in the chapter 4, I demonstrate that simultaneous expression of unphosphorylatable mutant forms of M18BP1 and HJURP leads to their premature centromere targeting, resulting in essentially complete reconstitution of CENP-A assembly.

The requirement and sufficiency of these two targets defines a two-step inhibitory mechanism in which Cdk1/2 are directly targeting both assembly factors (Figure 1.6 and Chapter 4 of this thesis). This dual level control ultimately allows for a strict cell cycle coupled timing of CENP-A assembly. Recently, another kinase, Plk1, was shown to act as a positive regulator of CENP-A deposition (McKinley and Cheeseman, 2014). Its localization to G1 centromeres and contemporaneous phosphorylation of M18BP1 proved to be important for robust recruitment of the M18 complex to G1 centromeres. Interestingly, Plk1 activity is necessary for both canonical and premature (G2 phase) deposition of CENP-A, indicating the requirement of positive phospho-signaling at all cell cycle stages. Therefore, the strict cell cycle coupling of CENP-A loading is achieved through negative Cdk1 and 2-dependant signals, restricting assembly to G1 while positive signals, such as Plk1 are needed to stimulate assembly (Figure 1.6).

While CENP-A assembly is uncoupled from DNA replication in most eukaryotes, in fission yeast and plants, CENP-A assembly occurs in premitotic G2 phase (Lando et al., 2012; Lermontova et al., 2006), although the molecular details remain elusive. Another outstanding question is assembly control in Drosophila. While G1 phase is the major cell cycle window where CENP-A assembly occurs (Lidsky et al., 2013; Schuh et al., 2007), in Drosophila somatic cell lines, some degree of assembly also takes place in other phases, notably in mitosis (Lidsky et al., 2013; Mellone et al., 2011). However, in neuroblasts, within the in vivo context of the organism, CENP-A assembly remains G1-restricted (Dunleavy et al., 2012). Rather than indicting a fundamentally different logic of control, these differences likely reflect physiological differences in the efficiency of inhibition by the cell cycle machinery, as artificially achieved in human cells.

In sum, a picture emerges where different mechanisms have evolved, all of which tie the CENP-A assembly machinery to the cell cycle.

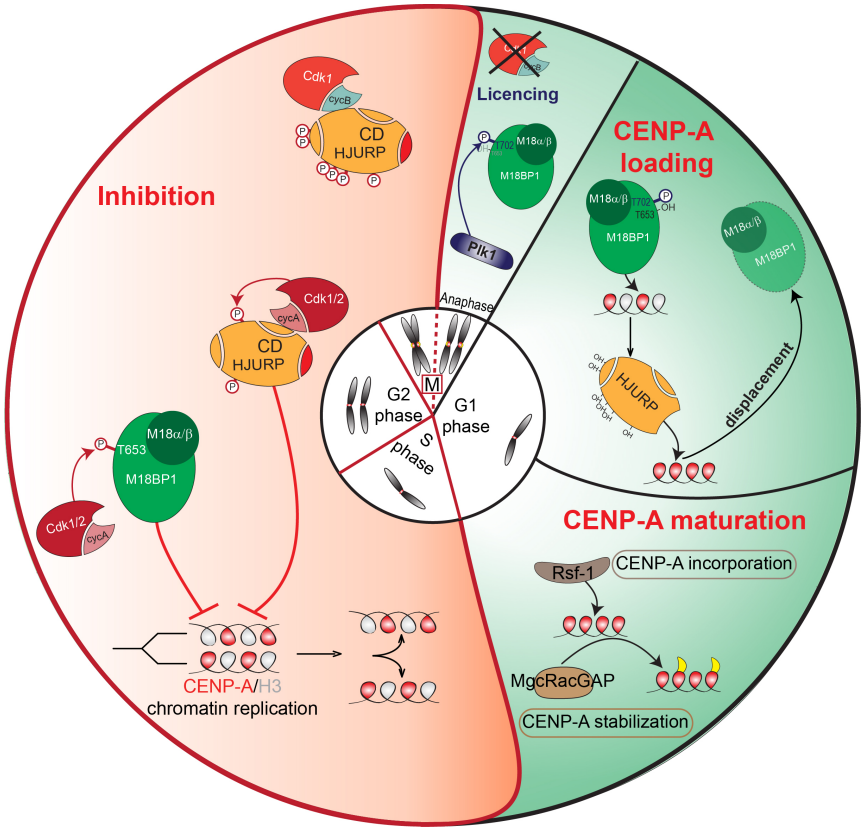

Figure 1.6 Overview of mechanisms ensuring cell-cycle coupled CENP-A assembly. CENP-A deposition is restricted to early G1 phase by the Cdk1/2 based phosphorylation of two key loading factors, M18BP1 and HJURP. During mitosis, positive regulation takes place in a form of licencing phosphorylation of M18BP1 by Plk1. Upon mitotic exit, negative regulation is alleviated and CENP-A assembly initiates. An additional step of ´´maturation´´ may be necessary in order to stabilize newly-loaded pool of CENP-A and to normalize CENP-A levels.

1.13 Centromere as a paradigm for epigenetic inheritance

At the peak of the Modern Synthesis, phenomena that were not promptly explained by Mendelian genetics were referred to as ´´soft inheritance`` (Mayr, 1980), and often were cast aside as a troublesome aberrations. However, it remained undeniable that the notion of genes as sole unis of inheritance is irreconcilable with heritability of all traits. Today, heritable traits whose propagation doesn't solely rely on nucleotide sequence are called epigenetic traits.
Heritable systems, whether genetic or epigenetic, adhere to some basic principles that include (1) the ability to survive through key steps of the cell cycle such as DNA replication, transcription and mitosis, (2) have the capacity to drive template-directed duplication and (3), the duplication of the mark is regulated such that each molecule gives rise to an equal number of copies in synchrony with cell division (Gómez-Rodríguez and Jansen, 2013).

The centromere, with its primary molecular determinant, the CENP-A histone, represents an extreme example of an epigenetically encoded trait. Today, a large body of evidence show that the defining feature of centromeres is the presence of CENP-A nucleosomes (Mendiburo et al., 2011), as has been hypothesized for many years (Warburton et al., 1997). Centromeric CENP-A is stably and quantitatively propagated through mitotic and meiotic divisions (Dunleavy et al., 2012; Jansen et al., 2007; Raychaudhuri et al., 2012), with the only detectable loss of existing molecules occurring through replicative dilution (Bodor et al., 2013; Dunleavy et al., 2011; Falk et al., 2015; Jansen et al., 2007). Along with the case of neocentromeres (Amor et al., 2004; Capozzi et al., 2009; Knegt et al., 2003; Tyler-Smith et al., 1999; Ventura et al., 2004; Wandall et al.,

1998), these studies demonstrate that the centromere is not only epigenetically defined, but also epigenetically inherited, thus serving as an example of transgenerational epigenetic inheritance. Additionally, loss of CENP-A during mitosis and meiosis renders centromere dysfunctional, showcasing the absolute requirement for CENP-A-based propagation of centromere, whereby existing CENP-A molecules serve as a template for assembly of new ones (Black et al., 2007; Blower and Karpen, 2001; Buchwitz et al., 1999; Fachinetti et al., 2013; Henikoff et al., 2000; Howman et al., 2000; Raychaudhuri et al., 2012; Régnier et al., 2005; Stoler et al., 1995; Talbert et al., 2002). Furthermore, assembly of nascent CENP-A at centromeres is strictly coupled to mitotic exit in animal cells (Bernad et al., 2011; Hemmerich et al., 2008; Jansen et al., 2007; Schuh et al., 2007; Silva et al., 2012), and regulated through the fundamental machinery driving cell cycle progression (McKinley and Cheeseman, 2014; Müller et al., 2014; Silva et al., 2012; Stankovic et al., 2017; Wang et al., 2014). In chapter 4 of thesis, I demonstrate that human cells utilize a dedicated Cdk-based regulation to limit the timing of CENP-A assembly to a discrete temporal window. Curiously, the two-step mechanism I propose to operate in this case doesn't differ tremendously from the one utilized to drive propagation of DNA molecule across cell divisions. Like in a genetic system, propagation of CENP-A containing domain requires the action of a ´´licensing´´ factor that will prepare chromatin for CENP-A duplication. In the genetic inheritance system this takes form in the assembly of pre-replicative complex (pre-RC), whereas in CENP-A based one is performed by the M18 complex. The action of the M18 complex sets the stage for incorporation of nascent CENP-A molecules, driven by its dedicated chaperone HJURP. Since HJURP acts as the seed-carrier that allows self-propagation of epigenetic mark it could be considered to be a ´´writer´´ of

centromeric epigenetic code, similar to DNA polymerase that incorporates nascent nucleotides based on the information provided by parental DNA molecules. Interestingly, chromatin disassociation of the ´´licensing´´ factor driven by the onset of heritable mark replication is another common feature between DNA and CENP-A based inheritance (chapter 4 of this thesis). Importantly, the molecular switch that controls the propagation of the centromere and DNA is essentially the same; low Cdk activities promote the licensing of the chromatin for the next round of DNA replication (Mailand and Diffley, 2005), concomitant with the temporal window in which the assembly of CENP-A is occurring (Jansen et al., 2007; Silva et al., 2012). Subsequently, DNA replication is triggered and CENP-A assembly is switched off as Cdk activities start to raise along the cell cycle.

In sum, the CENP-A molecule fulfills all the basic properties of a heritable epigenetic mark: stability, duplication and regulation, which allow for inheritance of an epigenetically encoded centromere.

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CHAPTER 2

Cdk1/2 dependent regulation of the Mis18 complex

This chapter contains sections of the publication: Ana Stankovic, Lucie Y. Guo, João F. Mata, Dani L. Bodor, Xing-Jun Cao, Aaron O. Bailey, Jeffrey Shabanowitz, Donald F. Hunt, Benjamin A. Garcia, Ben E.Black, Lars E.T. Jansen. A Dual Inhibitory Mechanism Sufficient to Maintain Cell-Cycle-Restricted CENP-A Assembly, *Molecular Cell*, 65.2 (2017), 231–46

Abstract

Propagation of centromeric chromatin occurs upon mitotic exit, in a process which is negatively regulated through Cdk1 and Cdk2 activities, and requires an orchestrated action of several CENP-A loading factors. Amongst them is the Mis18 complex, which operates as the most upstream factor necessary for the onset of CENP-A propagation. In this chapter, I will focus on the contribution of the Mis18 complex to the regulation of CENP-A assembly, with a particular emphasis on the largest member of the Mis18 complex, M18BP1.

Introduction

2. The Mis18 complex centromere targeting is required for CENP-A propagation across species

The founding members of what we today call the Mis18 complex, were initially discovered in *S. pombe*, as part of a genetic screen which aimed to identify novel factors involved in proper mitotic progression (Hayashi et al., 2004). Two of these factors, Mis16 and Mis18 were found to act as upstream factors involved in centromere recruitment of CENP-A (known as Cnp1 in fission yeast), while simultaneously contributing to kinetochore targeting of $Mis6^{CENP\text{-}I}$, $Mis15^{CENP\text{-}N}$, and Mis17. Mis16 shares ~50% sequence identity with the human general histone chaperones RbAp46/48 while Mis18, although not harbouring recognizable protein domains, has two homologues in humans, Mis18α and Mis18β. Whereas Mis16 and Mis18 formed a complex, resulting in co-dependency for their centromeric recruitment, no physical association was found between $CENP\text{-}A^{Cnp1}$ and either of the Mis factors. In addition, these proteins displayed a dynamic localization pattern, being targeted to centromeres in telophase followed by

delocalization in metaphase, indicative of cell cycle regulated centromere targeting. The vertebrate lineage contains two paralogs, Mis18α and Mis18β (Fujita et al., 2007; Stellfox et al., 2016), which share a common localization pattern, diffusely nuclear prior to anaphase, followed by a highly-enriched and centromere-specific localization in telophase. Interestingly, the human homologue of *S. pombe* Mis16, RbAp46/48, was found to associate (to a limited extent) with Mis18α in human cells, highlighting the functional conservation of the Mis18 complex organizations from fission yeast to human. In addition to Mis18α/β, Mis18 binding protein (M18BP1) was identified as an additional core component of the human Mis18 complex, sharing a common dynamic localization pattern with other Mis18 subunits, being transiently targeted to telophase centromeres with diffuse nuclear localization in other stages of the cell cycle. Importantly, downregulation of any of the components of the Mis18 complex revealed mutual dependency for their centromere targeting, resulting in defective recruitment of nascent CENP-A to the centromere, leading to increased formation of micronuclei and misaligned chromosomes. No M18BP1 homologue was found in *S.pombe*, however Mis19, has been proposed to operate in a similar fashion as M18BP1, given the fact that is centromere localized during interphase, but not mitosis, and is involved in mediating the interaction between Mis16 and Mis18 as well as their centromeric recruitment, ultimately regulating the levels of CENP-A^{Cnp1} at the centromere (Hayashi et al., 2014). Homologues of M18BP1 have been identified in developing embryos of *C. elegans* (Maddox et al., 2007), Xenopus eggs extracts (Moree et al., 2011) and mouse embryonic stem cells (Dambacher et al., 2012) together with Mis18α in mouse embryonic fibroblast (Kim et al., 2012). Interestingly, *C. elegans* embryos apparently lack homologues of Mis18α/β; however, KNL-2 was identified to be a

functional homologue of M18BP1, based on their shared Myb/SANT domain and involvement in the regulation of CENP-A propagation (Maddox et al., 2007). In contrast to the fission yeast, human and mouse studies (Dambacher et al., 2012; Fujita et al., 2007; Hayashi et al., 2004), CeCENP-A was found to be co-enriched with KNL-2 in chromatin fractions of *C. elegans* embryos (indicative of their proximity on centromeres, but not of a direct interaction, as authors also stated). To this date, this remains the only study in which such association has been detected. In addition, KNL-2 is present at kinetochores throughout the cell cycle in embryogenesis (Maddox et al., 2007), which could be attributed to the additional role of CENP-A$^{CeCENP\text{-}A}$ in promoting the condensation of holocentric chromosomes in which the centromere is formed along the whole length of chromosome arms (Maddox et al., 2006). Likewise, the stable association of KNL-2 with kinetochores could in part explain its co-enrichment with CENP-A. It is plausible that this interaction might also be occurring in the case of monocentric organisms, however due to the limited amount of time that the Mis18 complex resides at the centromere in such cases, this interaction would be transient and therefore hard to detect. Efficient RNAi-based depletion of KNL-2 in *C. elegans* drives a striking loss of centromeric CENP-A, resulting in a complete kinetochore failure and halt of embryonic development. In Xenopus egg extracts, M18BP1 is present in two isoforms (M18BP1-1 and M18BP1-2) both of which are targeted to interphase centromeres, similar to human and *S. pombe* Mis18 proteins (Fujita et al., 2007; Maddox et al., 2007). However, M18BP1-1 is additionally present on mitotic chromosomes (similarly to *C.elegans* embryos (Maddox et al., 2007)), followed by delocalization form centromeres upon mitotic exit and re-association after ~1h upon entry into the interphase (Moree et al., 2011). Co-depletion of both M18BP1 isoforms leads to a dramatic reduction of

total centromeric CENP-A, identifying xM18BP1 as one of the key regulators of xCENP-A homeostasis. In Arabidopsis, a putative homologue of M18BP1 carrying a cogent SANTA domain was identified, and as in the previous cases, complete knock-out of Arabidopsis KNL-2 caused reduction in the amount of centromeric CENP-A, leading to mitotic and meiotic abnormalities, along with reduced growth and fertility (Lermontova et al., 2013). Arabidopsis KNL2 is recruited to centromeres throughout the cell cycle, with the exception of metaphase to mid-anaphase (Lermontova et al., 2013). In sum, although a certain degree of variability regarding the timing of Mis18 centromere recruitment exists across different model organisms, the absolute requirement for their centromeric association allowing propagation of CENP-A containing chromatin remains a common conserved feature. A prominent exception is the Drosophila lineage that lacks recognizable Mis18 protein homologues, and relies on a sole factor, CENP-A^{CID} specific chaperone Cal1, to propagate centromeric domain (Chen et al., 2014; Erhardt et al., 2008; Mellone et al., 2011) .

2.2 The role of the Mis18 complex at the centromere

The designation of the Mis18 complex as CENP-A ´´licencing´´ or ´´priming´´ factor was instigated by the identification of functional homologues of spMis18 in human cells (Fujita et al., 2007). Since at the time of this discovery the G1 restricted deposition of CENP-A was not established (Jansen et al., 2007), it was proposed that this complex (analogous to the licencing of DNA by the pre-RC complex) is targeted to the centromere prior to its replication, in order to licence the chromatin for the deposition of CENP-A, which was anticipated to occur concomitantly with the replication of general chromatin or during G2 phase (Shelby et al., 2000). Given the association of RbAp46/48, with the Mis18 complex, this

study proposed that the licencing role of the Mis18 complex might be achieved through modulation of the amount of acetylated histones at the centromere (Fujita et al., 2007; Hayashi et al., 2004). RbAp46 and RbAp48 are highly homologous histone chaperones that play key roles in establishing and maintaining chromatin structure. They are integral subunits of protein complexes that either add or remove acetyl moieties from histone H4, and are known to interact with histone acetyltransferase HAT1, an enzyme that acetylates histone H4 specifically at lysine residues 5 and 12 prior to their incorporation into nucleosomes during replication (Roth et al., 2001). Indeed, defects observed in Mis18 depleted cells could be reverted through inhibition of histone deacetylases (HDAC), consistent with the previous study suggesting that RbAp46/48 depletion in human cells impacts the capacity of CENP-A to associate with the centromeres (Hayashi et al., 2004). However, given the wide role of these histone chaperones, attributing a direct relationship between RbAp46/48 and centromere maintenance has been difficult to establish, since acetylation of H4 is required for general chromatin assembly, including centromeres (Shang et al., 2016). Therefore centromere defects observed by RbAp46/48 depletion could be due to the decreased rate of chromatin assembly in general. However, several studies suggested that histone acetyltransferases may be involved in maintenance of an active CENP-A domain and *de novo* kinetochore formation. In 2003, (Nakano et al., 2003) proposed that one of the requirements for establishment of an active centromere assembled on ectopic alphoid DNA sequence is reduced activity of histones deacetylases (HDAC), leading to increased levels of acetylated histone H3 and transcription of a marker gene, which correlated with the maintenance of the ectopic centromere. However, a subsequent study demonstrated that sole inhibition of HDAC by Trichostatin A (TSA) is

necessary but not sufficient for the long-term maintenance of ectopic centromere (Okamoto et al., 2007). In addition, acetyltransferases, including p300 and PCAF localize at functional, but not at inactive centromeres during mitosis (Craig et al., 2003). This putative connection between centromeric acetylation and CENP-A homeostasis was further potentiated by investigating the requirements for formation of ectopic kinetochores in human cells (Ohzeki et al., 2012). This study took advantage of the fact that diverse human cell lines display differential capacity to nucleate de novo kinetochore (Ohzeki et al., 2012). Whereas assembly of artificial kinetochore is efficient in HT1080 cells, it was not in other commonly used ones (HeLa, IG7 human fetal primary cells, hTERTBJ1 immortalized fibroblasts and U2OS osteosarcoma cells). The reason behind this was the favourable centromeric ratio between active chromatin marks (H3K9ac) and repressive ones (H3K9me3), in which active marks were present in higher amount in HT1080 cells compared to HeLa. Remarkably, tethering of histone acetyl-transferase domains of p300 or PCAF to stably chromosomally integrated alphoid array carrying tet-operator binding sites (tetO), was sufficient to recapitulate *de novo* kinetochore assembly in HeLa cells, by counteracting the spreading of repressive marks from neighbouring pericentric region driven by Suv39h1 activity. Importantly, depletion of Mis18α was partially rescued by tethering of HAT proteins to synthetic alphoid arrays, while loss of CENP-A was persistent on the endogenous centromere. Recently, a causal link between acetyltransferases, the Mis18 complex and propagation of centromeric chromatin was demonstrated (Ohzeki et al., 2016). The largest member of the Mis18 complex, M18BP1, interacts with the HAT KAT7/HBO1/MYST2 largely via it C-terminal tail (although N-terminal portion can also recruit a limited amount of KAT7), and recruits KAT7 to G1 centromeres (Figure

2.1). Knocking out KAT7 reduced centromeric CENP-A assembly and a combination of knocking out KAT7 and overproducing Suv39h1 synergistically perturbed chromosomal segregation, compromising centromere function. Consistently with the localization pattern of M18BP1, transient increase in the amount of H3K14 acetylation was detected at alphoid DNA in G1 phase, leading to the proposal that centromeric HAT recruitment forms a permissive chromatin state for deposition of nascent CENP-A, which occurs in a discrete time window, in early G1 phase (Ohzeki et al., 2012, 2016). This is important in light of the fact that the centromere is flanked by pericentromeric chromatin carrying the repressive H3K9me3 modification (Sullivan and Karpen, 2004), whose formation is mediated through H3K9-metyltrasferases (Clr4 in *S.pombe*, SU(VAR)3-9 in *Drosophila*, Suv39h1 and Suv39h2 in mouse and humans). HP1 proteins interact with Suv39, H3K9me3 modifications and to each other, contributing to the spreading of pericentric chromatin and silencing of adjacent gene expressions, as illustrated by position-effect-variegation (PEV) (Allshire et al., 1994; Muller, 1930; Talbert and Henikoff, 2006). Since excessive heterochromatin spreading can inactivate centromere function (Cardinale et al., 2009; Nakano et al., 2008; Ohzeki et al., 2012), a counterforce ensuing balance between active and repressive chromatin marks is achieved by transient association of histone-acetyltransferases with the centromere, which occurs concomitantly with CENP-A deposition. On the other hand, force-targeting of HATs to centromeres lead to spreading of the CENP-A containing chromatin domain, showcasing that, under certain condition, centromeric chromatin, harbouring active histone marks can invade neighbouring pericentric chromatin, thus possibly compromising its function in sister chromatin cohesion (Hahn et al., 2013; Oliveira et al., 2014; Peters et al., 2008). Therefore, a dynamic equilibrium between active and

repressive histone marks drive the formation and protection of the active centromere, with the M18 complex playing an integral role in temporally restricted maintenance of active histone marks at the centromere. In addition to this function, the Mis18 complex promotes recruitment of the CENP-A specific chaperone HJURP to G1 centromeres (Barnhart et al., 2011; Pidoux et al., 2009; Williams et al., 2009), which deposits nascent CENP-A molecules, thus perpetuating stable centromere inheritance. In human cells, both Mis18α and Mis18β interact with HJURP through their conserved C-terminal coiled-coli domains (Figure 2.1). These domains mediate formation of a Mis18 oligomer (heterotetramer or hexamer) consisting of at least two homodimers of Mis18α and Mis18β, forming an interface which is recognized by the centromere targeting domain 1 of HJURP (HCTD1) (Nardi et al., 2016; Pan et al., 2017). This interaction appears to be negatively regulated through the action of (at least) Cdk1, which phosphorylates HJURP during mitosis, rendering it unable to interact with the Mis18 proteins (Wang et al., 2014). Conversely, HJURP can interact with Mis18α/β oligomer in chromatin-free extracts of interphase cells, indicating that this interaction can occur while Cdk1/2 are active (Nardi et al., 2016). Therefore, the question regarding cell cycle control of this interaction requires further investigation. However, in chicken DT40 cells, HJURP strongly interacts with M18BP1, not with Mis18α (Perpelescu et al., 2015). Nevertheless, the common theme is that HJURP centromeric recruitment relies on the initial targeting of the Mis18 proteins, while the revers is not the case (provided sufficient amount of CENP-A nucleosomes at the centromere). Overall, the Mis18 complex has a dual role at the centromere, reflected in co-recruitment of histone acetyl-transferases on the one hand, and CENP-A specific chaperone HJURP on the other, thus

providing a crucial link between cell cycle progression and propagation of centromeric chromatin.

2.3 Mechanisms of centromere recognition by the Mis18 complex

Initial RNAi based depletion suggested CENP-A-independent recruitment of the Mis18 complex to the centromere (Fujita et al., 2007; Hayashi et al., 2004). However, this can be attributed to the extreme stability and long half-life of CENP-A nucleosomes, requiring extensive amount of time for their complete disappearance from the centromere. In addition, human centromeres display a substantial resistance to fluctuating amount of CENP-A nucleosomes. A reduction of CENP-A up to 1% of the initial levels is still sufficient to maintain a functional centromere, at least in the short term (Fachinetti et al., 2013). Consequently, a residual amount of CENP-A nucleosomes is a likely explanation for efficient localization of the human Mis18 proteins in CENP-A RNAi background. Consistently, depletion of CENP-A in *C. elegans* embryos compromised KNL-2 kinetochore recruitment (Maddox et al., 2007). Mis18 proteins are not part of the CENP-A prenucleosomal, or chromatin-bound complex, with the exception of *C. elegans* embryos (Foltz et al., 2006, 2009; Lagana et al., 2010; Maddox et al., 2007; Shuaib et al., 2010). Instead of directly recognizing CENP-A nucleosomes, M18BP1 interacts with a member of the CCAN network, CENP-C, which, in turn, directly recognizes centromeric CENP-A and whose centromere localization is dependent on CENP-A (Carroll et al., 2010). In mouse embryonic fibroblasts (Dambacher et al., 2012) and human cells (Carroll et al., 2010; Moree et al., 2011), depletion of CENP-C reduces the centromere targeting efficiency of Mis18 proteins and thus leads to decrease in the amount of CENP-A assembled at the centromere. In Xenopus egg extracts (Moree et al., 2011), mitotic localization of

M18BP1-1 requires CENP-C, however it proved dispensable for its interphase targeting. Moreover, in the absence of CENP-C, M18BP1-1 accumulates at interphase centromeres to higher levels, indicating the existence of a second, CENP-C independent mechanism for targeting M18BP1-1 to centromeres (Moree et al., 2011). Recently, (Shono et al., 2015) demonstrated that in human cells, CENP-I, another member of the CCAN network can recruit M18BP1, albeit less efficient compared to CENP-C. Since CENP-C operates upstream of CENP-I dictating its centromeric localization, this parallel interaction was proposed to reinforce M18BP1 recruitment to centromere. Interestingly, the conserved SANTA domain is dispensable for association between CENP-C and M18BP1, rather its N-terminal portion (1-383 aa) together with the C-terminal part (476-721) form an interaction interphase which recognizes the C-terminal tail of CENP-C (Lermontova et al., 2013; Stellfox et al., 2016) (Figure 2.1). The N-terminal portion of M18BP1 mediates the interaction with a conserved YIPPEE domain of Mis18α. Mis18α, in turn oligomerizes with Mis18β through their respective conserved C-terminal coiled-coiled domains, recruiting Mis18α to G1 centromeres (Nardi et al., 2016; Stellfox et al., 2016). Even though Mis18α and Mis18β share 29% identity between YIPPEE domains, they also contain several amino acid that are distinctively conserved within each paralog (Figure 2.1). These differences confer differential binding to centromere-localized substrates; whereas Mis18α binds to M18BP1, the Mis18β YIPPEE domain recognizes C-terminal portion of CENP-C (694-943 aa), which occurs only during early G1 phase, indicating a strong cell cycle control over this interaction (Stellfox et al., 2016). Likewise, centromeric recruitment of M18BP1 is negatively regulated through Cdk1/2 activities, which limits its centromeric recruitment until loss of Cdk1 activity in anaphase (Silva et al., 2012; Stankovic et al., 2017).

Recently, another kinase, Plk1, was shown to act as a positive regulator of CENP-A deposition. Its localization to G1 centromeres and contemporaneous phosphorylation of M18BP1 is important for robust recruitment of the M18 complex to G1 centromeres (McKinley and Cheeseman, 2014).

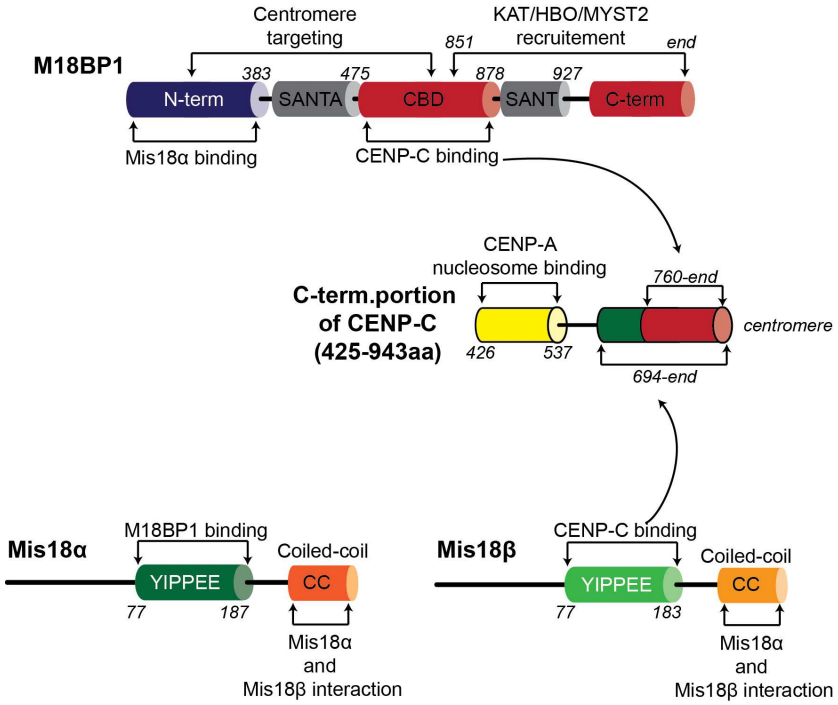

Figure 2.1 Domain structure of human M18BP1, Mis18α and Mis18β. M18BP1 contains two conserved domains, SANTA and SANT, neither of which is essential for centromere targeting. N-terminal portion (1-383aa) mediates interaction with Mis18α, whereas the central domain (475-878aa) contacts centromeric CENP-C. Mis18α and Mis18β are paralogues containing conserved YIPPEE domains. This domain shows 29% identity between Mis18α and Mis18β, and also contains residues that are unique and conserved in each paralogue across species. The differences within YIPPEE domain confer differential binding to substrates; Mis18α binds to N terminus of M18BP1, while Mis18β recognizes centromere-bound CENP-C. Both proteins contain conserved coiled-coils on C-terminal part, which facilitates interaction between Mis18α and Mis18.

2.4 Material and Methods

DNA constructs

GFP-M18BP1^{T653A} (pLJ649) was created by quick exchange PCR using GFP-M18BP1 as a template (pLJ415 (Silva et al., 2012)). An analogous procedure was employed to generate GFP-M18BP1T653D (pLJ699). For construction of GFP-M18BP1$^{T4A,\ T40A,\ S110A}$ a synthetic GeneStringTM (Invitrogen) was ligated to pLJ649 via PstI and XhoI ligation. CBdbd-GFP-M18BP1 (pLJ592) was generated by ligation of CBdbd fragment between GFP and M18BP1 of the GFP-M18BP1 plasmid (pLJ415) mRFP-M18BP1T653D (pLJ705) was created by NotI and AfeI replacement of GFP with pLJ287. CBdbd-mRFP-M18BP1/M18BP1^{T653D}/M18BP1^{T653E} (pLJ697, pLJ700 and pLJ642, respectively) were created by PCR insertion of CBdbd fragment from pLJ591 (CBdbd-GFP-HJURP) into pLJ534 (mRFP-M18BP1) or pLJ705 (mRFP-M18BP1T653D) or pLJ641 (mRFP-M18BP1T653E). PCR amplified CBdbd was subsequently fused to N-terminal portion of mRFP. mRFP-Mis18β was constructed by AfeI, AgeI and KpnI digestion of GFP-Mis18β (pLJ382) and subsequent ligation into mRFP expression plasmid (pLJ287). CBdbd-RFP-Mis18β was generated by SpeI and EcoRI digestion of GFP-Mis18β (pLJ382), followed by ligation of generated fragment into CBdbd-RFP-M18BP1 (pLJ697). pbabe-Puro-Mis18β was generated by AgeI and KpnI digestion of GFP-Mis18β (pLJ382) and ligation into pbabe-Puro plasmid (pLJ232). GFP-HJURP (pLJ380) and GFP-HJURP-ΔCLacI (pLJ632) were converted to GFP-HJURP^{AxA} (pLJ600) and GFP-HJURP^{AxA}-ΔCLacI (pLJ654) by quick exchange PCR replacing R276 and L278 by Alanine.

Cell lines

All human cell lines used were grown at 37°C, 5% CO2. Cells were grown in DMEM (Bio West) supplemented with 10% fetal bovine serum (FBS) (BioWest)), 2 mM glutamine, 1 mM sodium pyruvate (SP) (Thermo Fischer Scientific), 100 U/ml penicillin, and 100 µg/ml streptomycin, with the exception of HeLa HILO derived cell lines in which 10% tet-free (BioWest) FBS was used. HeLa HILO RMCE cell lines were a gift from E.V. Makeyev, Nanyang Technological University, Singapore, and contain a single genomic recombination which allows for the insertion of a tetracycline responsive expression cassette (Khandelia et al., 2011). The four lines outlined in Figure 2.5 were assembled as follows: HeLa HILO RMCE clone #10 (Khandelia et al., 2011), was transfected with (pLJ649) that constitutively drives GFP-M18BP1T653A expression. Positive clones were selected with 500 µg/ml of Neomycin (Gibco). A polyclonal population was sorted based on GFP fluorescence. For construction of GFP-Mis18β, CENP-A-SNAP stable cell line, parental HeLa CENP-A-SNAP #72 were infected with a Moloney murine leukemia retroviral delivery as previously described (Shah et al., 2004). Cells stably expressing CENP-A-SNAP and GFP-Mis18β were selected using 0,1 µg/mL of Puromycin (Calbiochem) for one week, followed by an additional selection with 1 µg/mL of Puromycin (Calbiochem). After reaching confluency, polyclonal cell population was single-cell sorted in a MoFlo High-Speed Cell Sorter (Beckman Coulter, USA). The resulting monoclonal lines were expanded and selected by fluorescence microscopy for expression level and localization.

DNA transfection

Transient transfection of HeLa and HEK293T was performed using Lipofectamine LTX (Invitrogen; Carlsbad, CA) according to manufacturer´s

instructions.

Phospho-specific anybody generation, application and phosphatase treatment

Phospho-specific rabbit antibody for M18BP1 was produced by immunization of 2 rabbits with phosphorylated peptide ((NH2-)CKAYILV (pT)PLKSRK (-CONH2)), and subsequent affinity purification of both sera (Innovagen AB, SE-22370 Lund, Sweden). 10^6 of transiently transfected HEK293T carrying either GFP-M18BP1 or GFP-M18BP1^{T653A} were lysed in buffer containing 75mM HEPES pH 7,5, 150mM NaCl, 2mM MgCl2, 0,1% NP-40, 5% Glycerol, 2mM EDTA supplemented with Roche complete protease and phosphatase inhibitors. Lysates were spun at 4°C for 5 min at 15,000 x g. Supernatants were either left untreated or 300 units of Lambda phosphatase was added. All samples were incubated for 30 min at 30°C. Reaction was stopped by addition of 4 x Orange sample buffer. For assaying Cdk dependent phosphorylation of M18BP1, transiently transfected Hek293T were treated with 100µM of Roscovitine for 30min, or treated for DMSO. Protein extracts were separated by SDS-PAGE and probed with pT653 and GFP (Chromotek) antibodies. Fluorescence activated cell sorting (FACS) for cell cycle profile was performed based on propidium iodide staining as described (Silva et al., 2012).

Cell synchronization

Double Thymidine-based synchronization was performed as described (Bodor et al., 2012). For Mitotic synchronization, 2,4 µM of EG5 inhibitor III Dimethylenastron-DMEIII (Calbiochem) was used for 24h. For synchronous mitotic exit, following DMEIII washout, HeLa and Hek293T were released for 5h and 7h, respectively.

SNAP quench-chase-pulse labelling

Cell lines expressing CENP-A-SNAP were pulse labeled as previously described (Bodor et al., 2012).

Immunofluorescence

Procedures are essentially as described (Bodor et al., 2012). Briefly, all cell lines were grown on glass coverslips coated with poly-L lysine (Sigma-Aldrich) and fixed with 4% formaldehyde (Thermo Scientific) for 10 min followed by permeabilization in 0.1% Triton X-100. HeLa cells were stained with anti-cyclin B1 (1:50; sc-245, Santa Cruz) and antiCENP-T (Barnhart et al., 2011). Secondary antibodies used were either FITC-conjugated anti-mouse (Jackson Immunoresearch Laboratories) or Dy680 conjugated anti-rabbit antibody (Rockland Immunochemicals). Cells were stained with DAPI (40, 6-diamidino-2 phenylindole; Sigma-Aldrich) before mounting in Mowiol.

Microscopy

Imaging was performed using a DeltaVision Core system (Applied Precision) inverted microscope (Olympus, IX-71), coupled to a Cascade2 EMCCD camera (Photometrics). Images (ranging from 512x512 to 1024x1024) were acquired at 1 x binning using a 100x oil objective (NA 1.40, UPlanSApo) with 0, 2 µm z sections.

2.5 Results

Recruitment of the Mis18 complex to the centromere is controlled by phosphorylation of M18BP1T653

Previously, we reported that a phospho-dead M18BP1 mutant in which 24 known phospho-sites are mutated to alanine, resulted in its premature centromere targeting (Silva et al., 2012), suggesting that at least one of these sites is regulated by Cdks. To pinpoint key residues in M18BP1 which may be responsible for its cell cycle dependent localization, we performed a conservation analysis of the M18BP1 protein, in which we evaluated the extent of conservation of the Cdk recognition motif. Based on this, we identified four putative Cdk motifs that are highly conserved among vertebrates, three of which are clustered close to the N-terminus of M18BP1 (T4, T40 and S110), while a fourth (T653) is located between the highly conserved SANTA and SANT domains (Maddox et al., 2007) (Figure 2.2A). To test the contribution of these residues in control of M18BP1 localization, we transiently transfected a HeLa CENP-A-SNAP cell line with a GFP-tagged M18BP1 construct carrying designated Alanine mutations. Mutation of all 4 sites to alanine leads to a loss of cell cycle controlled localization of M18BP1, resulting in the precocious recruitment of M18BP1^{4Ala} to G2 centromeres (Figure 2.2C). Mutations encompassing T4, T40 and S110 associated with the centromere to a higher level compared to wild-type protein, albeit to a lesser extent than M18BP1^{4Ala}. Interestingly, mutation of T653 alone was sufficient to result in premature centromere targeting of M18BP1 with a ~3-fold increase in G2 centromeric levels relative to wild type protein (Figure 2.2B). Importantly, M18BP1^{T653A} retained its capacity to localize to early G1 centromeres. We generated a phospho- and site-specific antibody against the T653 site and show that

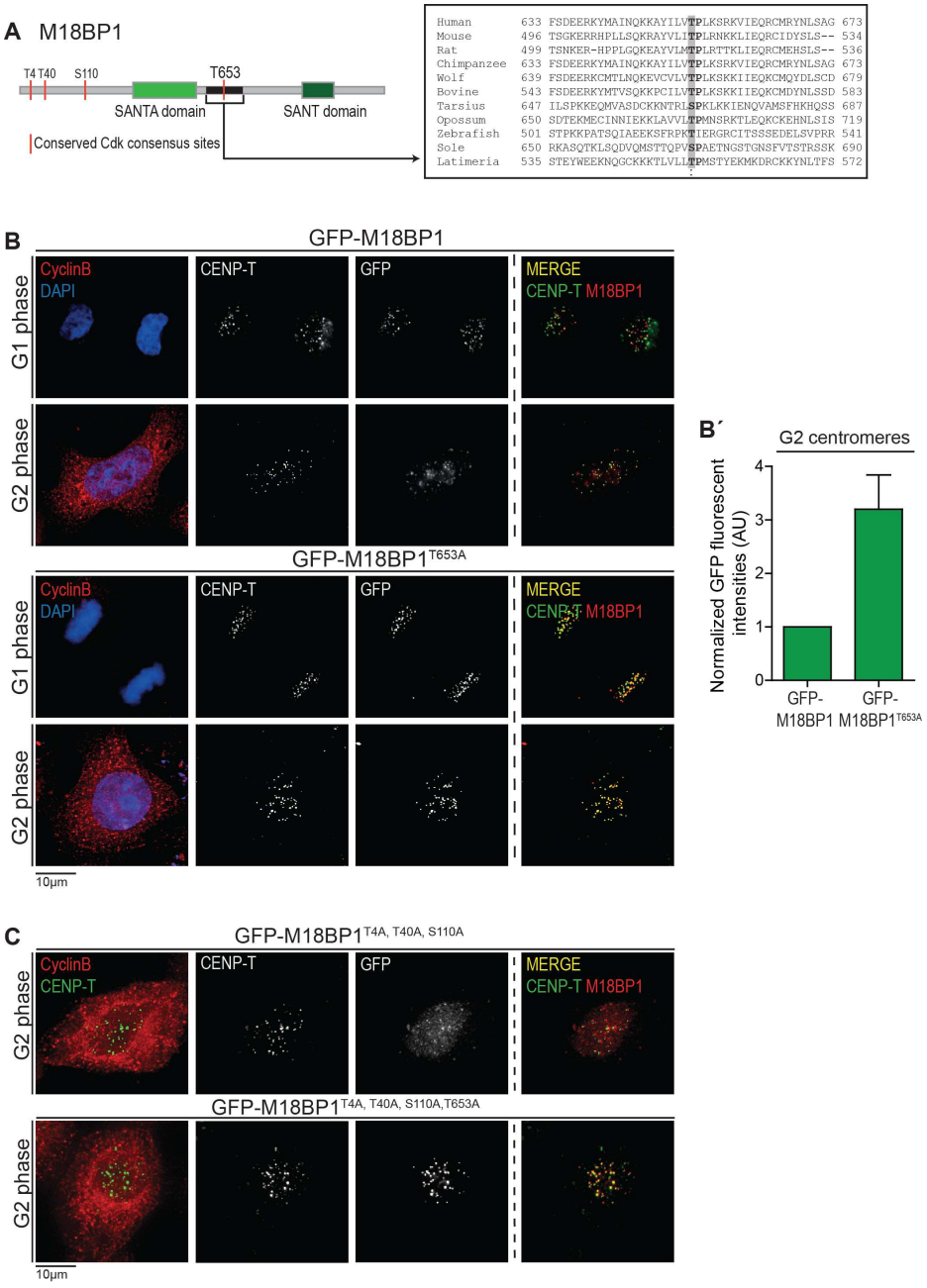

Figure 2.2 Cdk-mediated T653 phosphorylation of M18BP1 controls its centromere recruitment. (A) M18BP1 T653 is conserved amongst vertebrates. Left: Schematic of

Figure 2.2 Continued: M18BP1 protein. Relevant domains and conserved Cdk sites are indicated. Right: Conservation of human T653 residue across species. Conserved Threonine or Serine is highlighted in grey. (B) T653 residue controls cell cycle-dependent M18BP1 centromere recruitment. Indicated constructs were transfected into asynchronous HeLa cells 48hr prior to fixation, followed by counterstaining for cyclin B, CENP-T and DAPI to indicate G2 status, centromeres and DNA, respectively. (B´) Average centromeric GFP fluorescent signals from Cyclin B positive cells were determined using the Centromere Recognition and Quantification (CRaQ) method (Bodor et al., 2012c) and normalized to GFP-M18BP1. Error bars indicate standard error of the mean (SEM) from 3 replicates. (C) M18BP1^{T4,T40,S110} triple mutant is enriched at G2 centromeres, whereas quadruple mutant is strongly enriched at G2 centromeres. Constructs expressing M18BP1T4,T40,S110 or M18BP1T4,T40,S110,T653A were transfected into asynchronous HeLa cells 48hr prior to fixation, followed by counterstaining for cyclin B, CENP-T and DAPI to indicate G2 status, centromeres and DNA, respectively.

this residue is indeed phosphorylated in a cell cycle dependent manner. pT653 levels rise as cells accumulate in S/G2 and mitosis, correlating with increasing levels of Cdk1 and 2 activities (Figure 2.3A). A brief treatment of cells expressing GFP-M18BP1 with a Cdk1/2 inhibitor caused a strong reduction in phosphorylation of T653, suggesting that M18BP1 is a direct target of these kinases (Figure 2.3B).

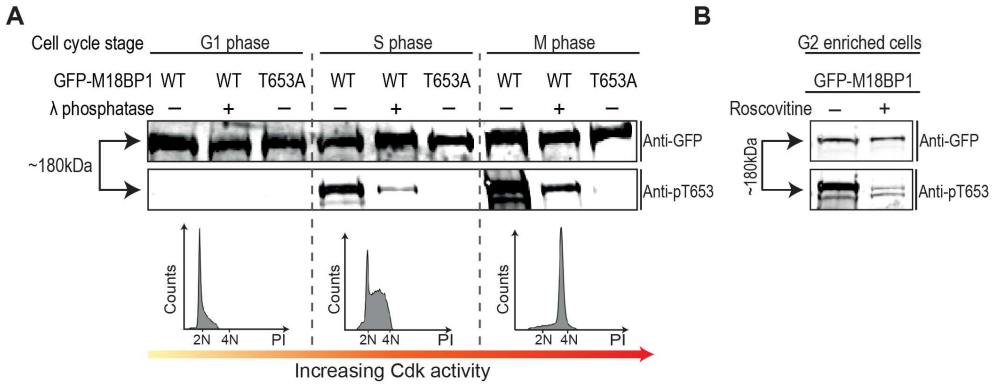

Figure 2.3 T653 is phosphorylated in a cell cycle dependent manner. (A) Hek293T cells were transiently transfected with GFP-Mis18BP1(WT) or GFP-Mis18BP1^{T653A} as a non-pho-

Figure 2.3 Continued: -sphorylatable control. 24h later, cells were synchronized in indicated cell cycle stages and lysed. Extracts were either left untreated or treated with lambda phosphatase, separated by SDS-PAGE followed by immunoblotting with indicated antibodies (see also supplemental experimental procedures). Apparent molecular weight is indicated. Cells were assayed for cell cycle position by FACS using propidium iodide (PI) to indicated DNA content. (B) T653 is phosphorylated by Cdk1/2. Hek293T cells were transiently transfected with GFP-Mis18BP1 and enriched in G2 phase by a single thymidine block followed by 7h of release. 30min before fixation, cell were treated with 100µM Roscovitine. Extracts were separated by SDS-PAGE followed by immunoblotting with anti-GFP and anti-pT653 antibodies.

To further investigate the impact of premature M18BP1 centromere recruitment, we assayed for premature assembly of CENP-A-SNAP in 24h time window following GFP-M18BP1^{T653A} expression. Despite of its centromere localization, no precocious CENP-A assembly was observed (Figure 2.4A). Consistently, artificial centromere targeting of M18BP1 in G2 phase, achieved by expression of a translation fusion between the DNA binding domain of CENP-B (CBdbd) which binds specifically to centromeric α-satellite DNA, and otherwise wild-type M18BP1, was also not sufficient to induce unscheduled CENP-A assembly (Figure 2.4B). Likewise, G2 tethering of M18α also failed to assemble CENP-A under high Cdk activities (Figure 2.4C). One possibility explaining the inability of M18BP1 to stimulate G2 phase CENP-A assembly is that the amount of protein accumulated at G2 centromeres is far below of the one required to instigate CENP-A deposition.

To test this, we have measured and compared GFP-M18BP1^{T653A} fluorescent intensities present at G2 and G1 centromeres, in a HeLa HiLo cell line stably expressing low levels of this protein (Figure 2.5A). The extent of centromere-localized GFP-M18BP1^{T653A} was not significantly different between two cell cycle stages, indicating complete uncoupling of

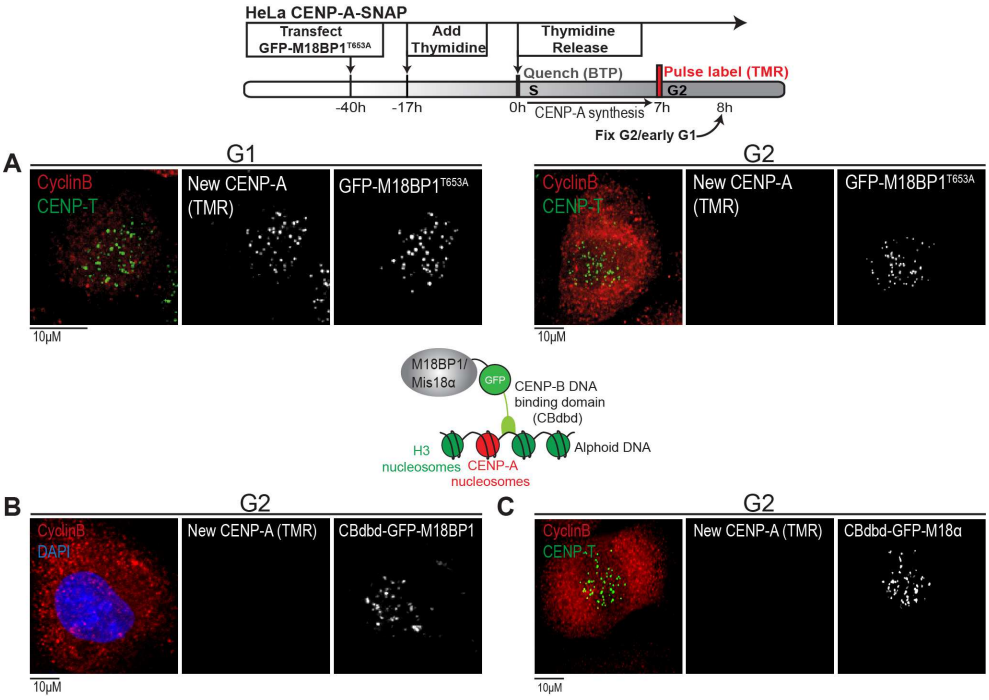

Figure 2.4 Transient premature centromeric targeting of the M18 complex proteins is not sufficient to alleviate Cdk1/2-dependent inhibition of CENP-A assembly. (A) HeLa CENP-A-SNAP were transiently transfected with GFP-M18BP1^{T653A} construct. 48h post-transfection, CENP-A assembly was assayed using SNAP TMR-labeling of its S phase synthesized pool. Following fixation, cells were counterstained for cyclin B and CENP-T to indicate G2 status and centromeres, respectively. (B,C) Top: Schematic of relevant domains in centromere targeted M18BP1 or Mis18α. HeLa CENP-A-SNAP cells were transfected with indicated constructs. 48h post-transfection, S phase synthetized pool of CENP-A was labelled using SNAP TMR. Following fixation, cells were counterstained for cyclin B and DAPI to indicate G2 status and DNA (B) or cyclin B and CENP-T to indicate G2 status and centromeres, respectively (C).

M18BP1 centromere targeting from cell cycle control (Figure 2.5A´). Interestingly, we did observe infrequent and low-efficient premature CENP-A assembly under constitutive expression of M18BP1^{T653A}, indicating that under these circumstance, the cell cycle inhibition can be partially

overridden (Figure 2.5A´´). However, the amount of prematurely deposited CENP-A represents only a small fraction of the one deposited in G1 phase, indicating that M18BP1 cannot be the sole factor responsible for the cell cycle-dependent mechanism of CENP-A assembly.

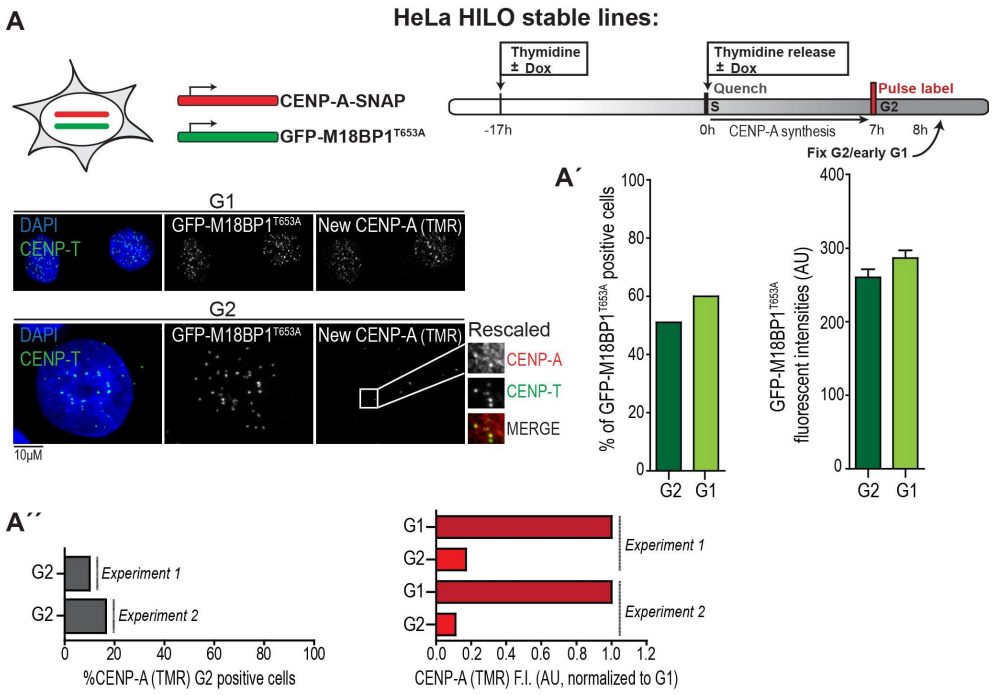

Figure 2.5 Constitutive centromeric targeting of M18BP1^{T653A} induces a low level of premature CENP-A assembly. (A) Top: Schematic representation of Hela HILO cells constitutively expressing low levels of CENP-A-SNAP (red), with stable expression of GFP M18BP1^{T653A} (green) Bottom: Representative images of cells described above. Following release from a single Thymidine block, S-phase synthetized pool of CENP-A was labelled using SNAP TMR. Following fixation, cells were counterstained for CENP-T and DAPI to indicate centromeres and DNA, respectively. Cell cycle status was determined by measuring total DAPI area (see Material and Methods). (A´) Left: Quantification of frequency of centromere-localized GFP-M18BP1^{T653A} in G2 and G1 phase in Hela HILO cells. Right: Quantification of GFP-M1BP1^{T653A} fluorescent signals present at G2 and G1 centromeres in Hela HILO cells. Average GFP-M1BP1^{T653A} signals from G2 centromeres were normalized to

Figure 2.5 Continued: respective G1 centromeres and corrected for centromere number (assuming signal intensity per focus represents 1 and 2 centromeres in G1 and G2, respectively). (A´´) Quantification of frequency of premature CENP-A loading in G2 cells expressing GFP-M18BP1^{T653A}. Right: Quantification of CENP-A-SNAP (TMR) fluorescent signal intensities in a manner analogous to the one described in (A´) 2 replicate experiments are shown.

Mis18α can form a complex with M18BP1 or Mis18β in G2 phase of the cell cycle

To test the involvement of Cdk1/2 in regulating the interaction amongst subunits of the M18 complex, we looked at the capacity of our M18BP1^{T653A} mutant to form a complex with its direct binding partner Mis18α. Using a HeLa cell line stably expressing GFP-Mis18α we assayed for its centromeric recruitment driven by the expression of M18BP1^{T653A}. Indeed, the M18BP1^{T653A} mutant co-recruited Mis18α to G2 centromeres, indicative of ongoing Mis18 complex formation independent of T653 phosphorylation (Figure 2.6A). To test whether M18BP1 phosphorylation of T653 results in disruption of the Mis18α interaction, we expressed a translational fusion of wild type or mutant M18BP1 to the CBdbd in cells synchronized in G2 phase. Forced recruitment of M18BP1 to centromeres leads to strong co-recruitment of Mis18α to G2 centromeres, suggesting that the Mis18 complex can form under inhibitory Cdk activity, at least at this stage of the cell cycle, although not in mitosis as observed previously (McKinley and Cheeseman, 2014) (Figure 2.6B). Similarly, forced recruitment of a phosphomimetic M18BP1^{T653D} mutant is capable of co-recruitment of Mis18α (Figure 2.6B). Thus, we find that mutation of the T653 residue does not disrupt the M18BP1/Mis18α interaction. Rather, its phosphorylation prevents centromere targeting of the Mis18 complex in G2 phase until mitotic exit when Cdk1/2 activities are low. Similarly to M18BP1 tethering,

artificial placement of Mis18β resulted in a strong enrichment of Mis18α to G2 centromeres.

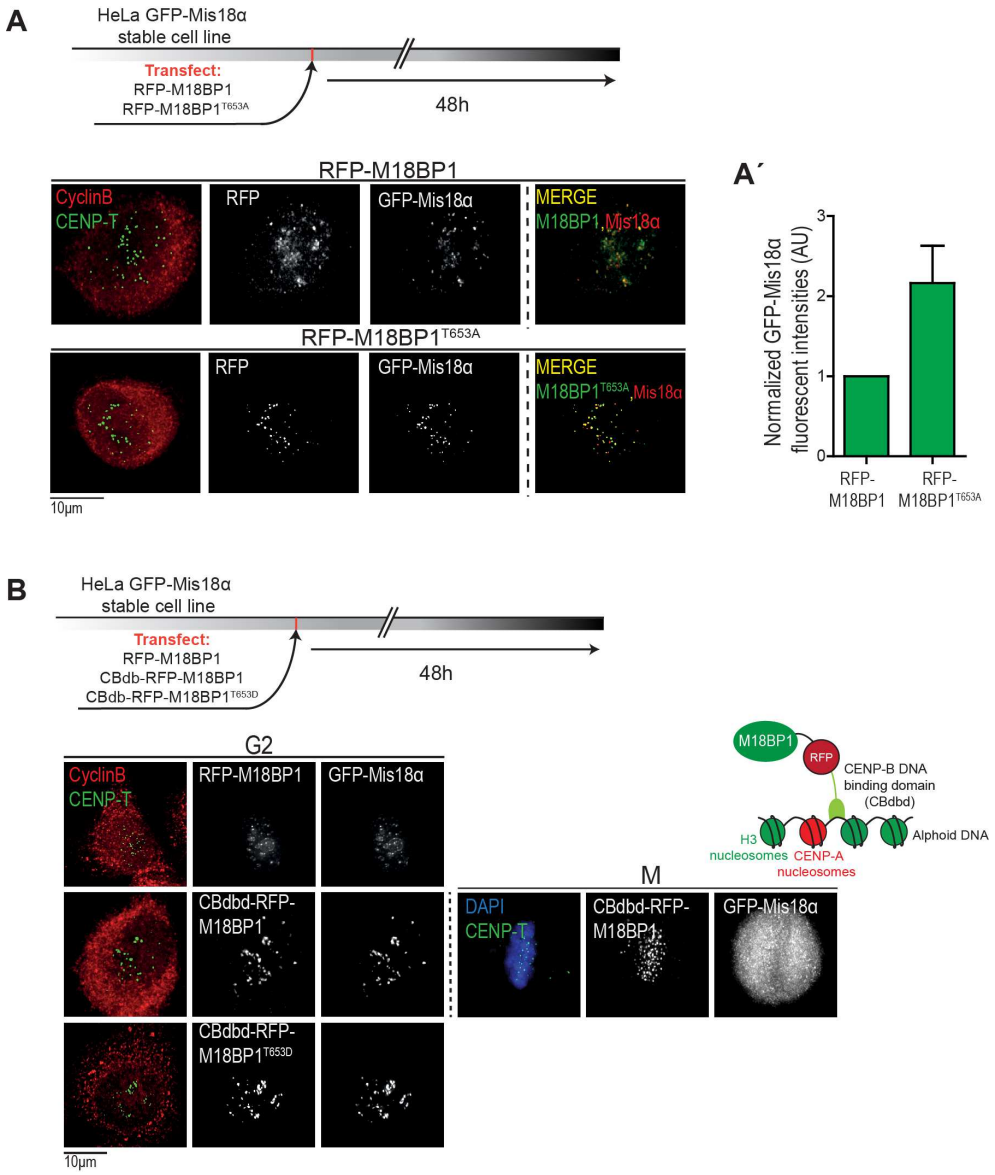

Figure 2.6 Cell cycle control of M18BP1/Mis18α complex formation. (A) T653 residue in M18BP1 does not determine Mis18 complex formation. Left: Asynchronous HeLa cells

Figure 2.6 Continued: stably expressing GFP-Mis18α were transfected with constructs expressing RFP-M18BP1 or RFP-M18BP1T653A 48hr prior to fixation, followed by counterstaining for cyclin B and CENP-T to indicate G2 status and centromeres. (A´) Average centromeric GFP fluorescent signals from Cyclin B positive cells were determined from 3 replicate experiments. Intensities were normalized to GFP-M18BP1. Error bars indicate standard error of the mean (SEM). (B) M18BP1/Mis18α complex formation is not inhibited by Cdk activity in G2 phase. Asynchronous HeLa-GFP-Mis18α were transfected with constructs expressing RFPM18BP1, CBdbd-RFP-M18BP1 or CBdbd-RFP-M18BP1T653D 48hr prior to fixation, followed by counterstaining for cyclin B and CENP-T to indicate G2 status and centromeres. To enrich for mitotic stages, cells were treated with Nocodozole for 5h.

Next, we wanted to explore whether M18BP1 is the driving factor in M18 complex formation. We demonstrated that in G2 phase, M18BP1 co-recruits Mis18α, but paradoxically, we did not observe the same behavior in reciprocal experiments (Figure 2.7). Similarly, artificial targeting of Mis18β did not co-recruit M18BP1 to G2 centromeres (Figure 2.7). These results suggest that M18BP1 localization at the centromere is a pre-requisite for Mis18α/β centromere targeting, which is in agreement with the fact that M18BP1 localize to centromeres already during mitosis (McKinley and Cheeseman, 2014), possibly providing a scaffold for Mis18α/β centromere targeting.

Mis18β cannot form a complex with M18BP1 in G2 phase of the cell cycle

Given that artificial tethering of M18BP1 is sufficient to recruit Mis18α to G2 centromeres (Figure 2.6), we wanted to explore if Mis18β, a direct binding partner of Mis18α, behaves in a similar manner. Interestingly, tethering of M18BP1 never resulted in Mis18β recruitment, in agreement with (Stellfox et al., 2016) (Figure 2.7), whereas tethering of Mis18α caused partial

premature recruitment of Mis18β. Robust premature Mis18β centromere localization were achieved either through Roscovitine treatment of G2

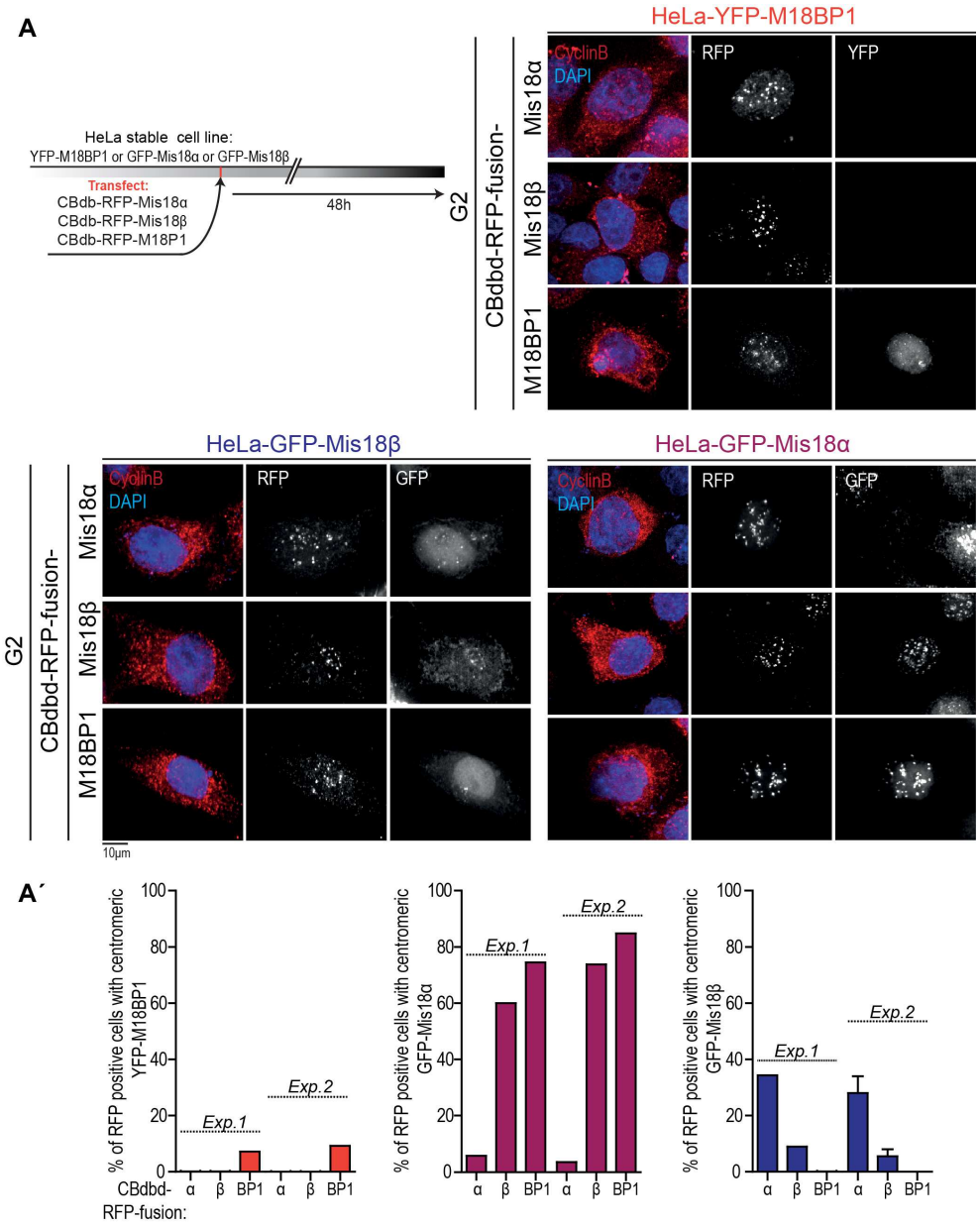

Figure 2.7 Cell cycle control of the Mis18 complex formation. (A) HeLa cells stably expressing YFP-M18BP1, GFP-Mis18α or GFP-Mis18β were transfected with indicated

Figure 2.7 Continued: constructs. 48h post-transfection, cells were fixed and followed by counterstaining for cyclin B and DAPI to indicate G2 status and DNA respectively. (A´) Quantification of frequency of Cyclin B positive cells having centromere-localized RFP colocalizing with either YFP or GFP signal. 2 replicate experiments are shown.

synchronized cells (Figure 2.8), or by simultaneous expression of mutant HJURP protein which mediates unscheduled CENP-A deposition (Figure 2.9A, B). As I will describe in the chapter 3, we identified a mutant of HJURP, named HJURP^{AxA}, which is capable of driving premature CENP-A deposition in G2 phase. This mutant is hypo-phosphorylated even in the presence of high Cdk1/2 activities, which renders it active in CENP-A deposition.

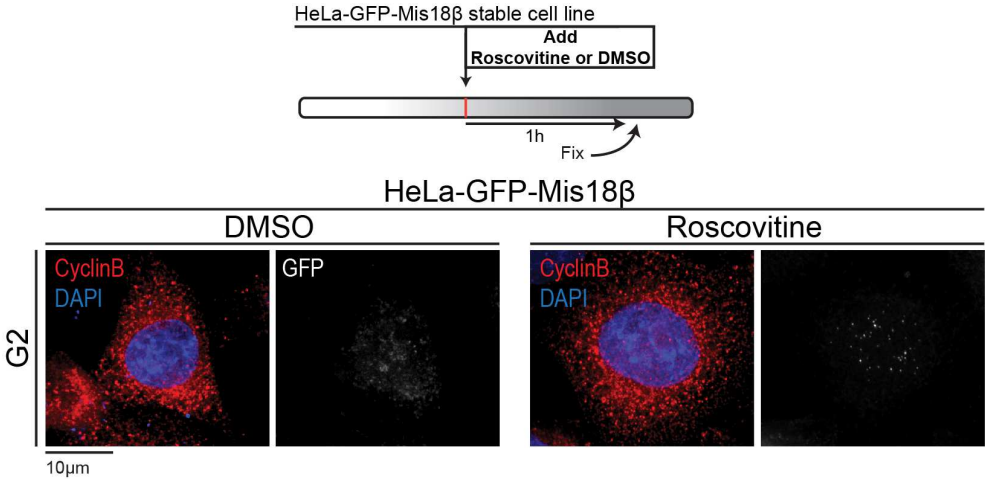

Figure 2.8. Roscovitine treatment induces precocious Mis18β centromere targeting. HeLa cells stably expressing GFP-Mis18β were treated for 1h with Roscovitine or DMSO as a control. Following fixation, cells were counterstained for cyclin B and DAPI to indicate G2 status and DNA respectively

Surprisingly, increasing the residence time of M18BP1 through over expression or by Cbdbd tethering specifically at G1 centromeres, decreased the efficiency of centromeric recruitment of Mis18β, whereas

Mis18α remained unaffected (Figure 2.10), suggesting Mis18β is dynamically recruited and turned over as part of the CENP-A assembly process.

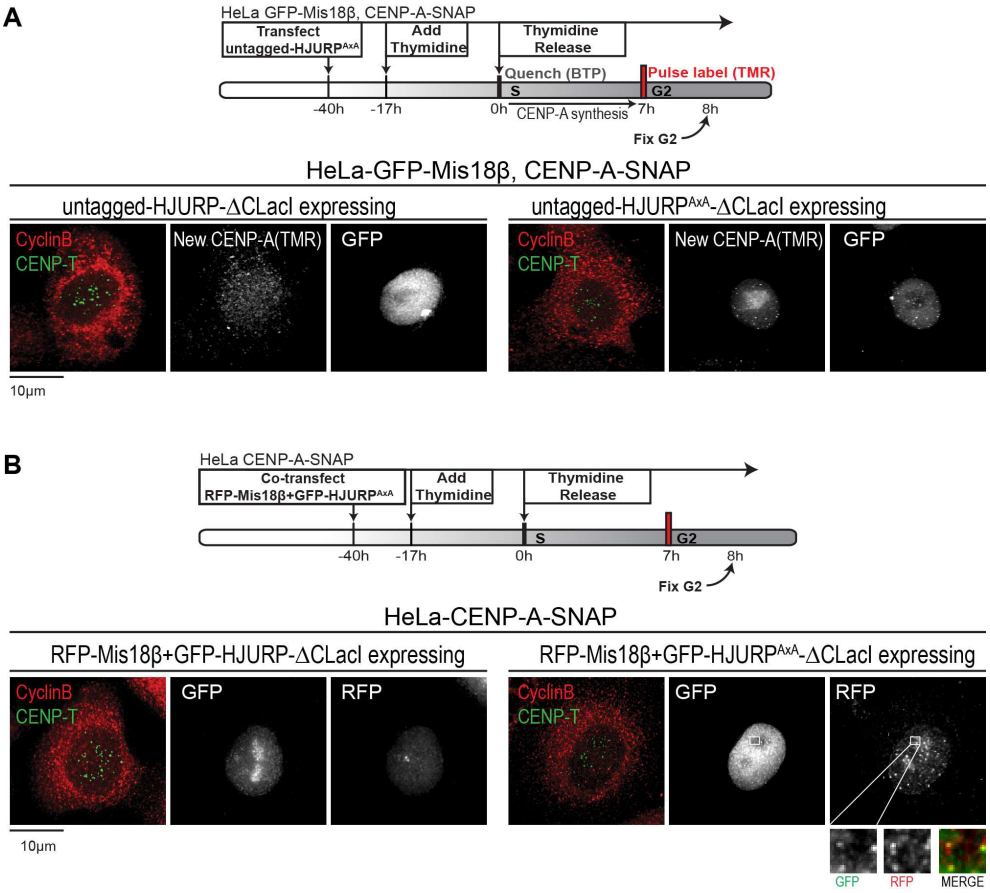

Figure 2.9 Premature Mis18β centromere targeting coincides with precocious CENP-A assembly driven by the expression of HJURP mutant. (A) HeLa-GFP-Mis18β cells were transiently transfected with indicated constructs.48h after transfection, S phase synthetized pool of CENP-A was labelled using SNAP TMR, followed by counterstaining for cyclin B and CENP-T to indicate G2 status and centromeres respectively. (B) HeLa-CENP-A-SNAP cells were co-transfected with indicated constructs. 48h post-transfection, cells were fixed and counterstained for cyclin B and CENP-T to indicate G2 status and centromeres respectively.

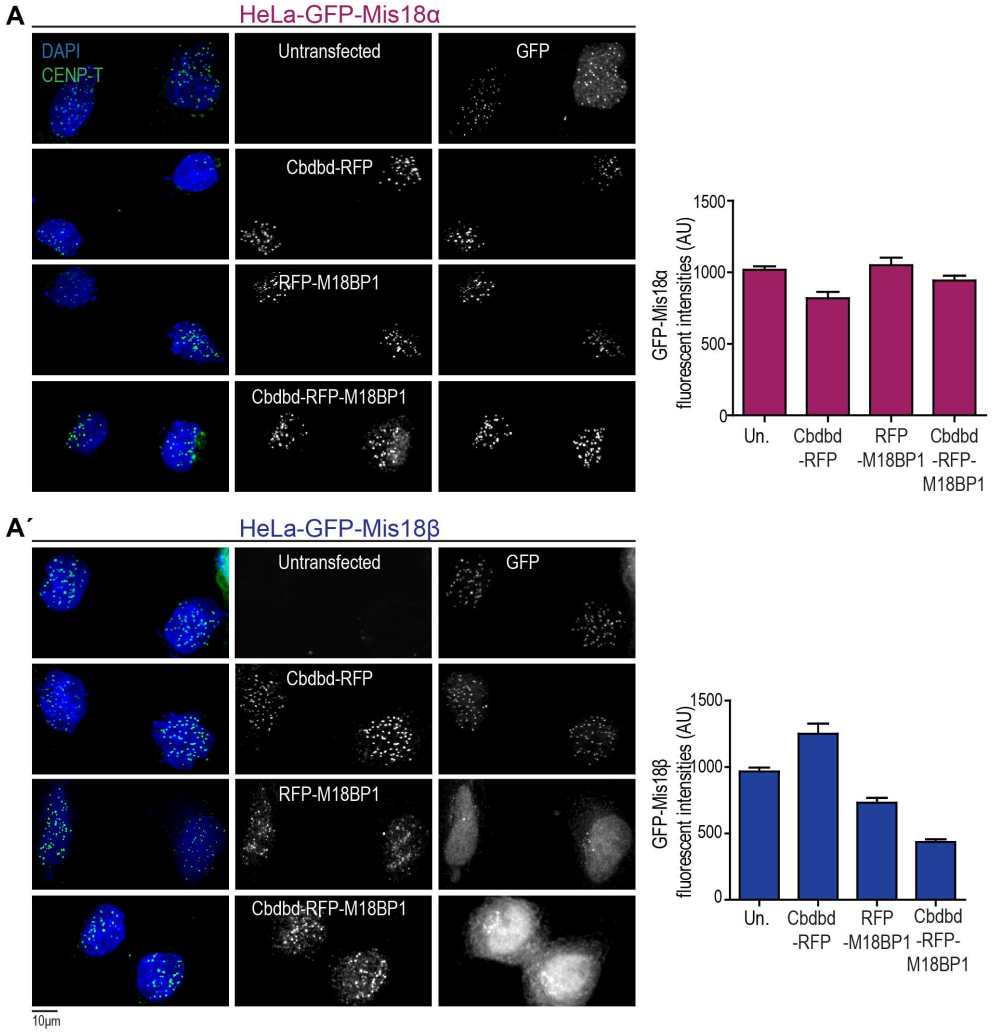

Figure 2.10 An apparent negative correlation in centromere occupancy between M18BP1 and Mis18β. (A) HeLa GFP-Mis18α or HeLa GFP-Mis18β (A´) cells were transfected with indicated constructs, and synchronized in mitosis by an overnight treatment with Eg5 inhibitor (DMEIII). Newly synthesized CENP-A pool was quenched in mitosis, followed by 5h of release in early G1 when nascent CENP-A-SNAP was labeled with TMR (G1 specific pool). GFP positive cells were selected and CENP-A TMR fluorescent intensities were determined using CRaQ, with the exception of the untransfected control where all cells were analyzed (A and A´).Error bars indicate standard error of the mean (SEM) of 3 experimental replicates.

2.6 Discussion and Conclusions

In this chapter, I describe our demonstration that centromere recruitment of the Mis18 complex is negatively regulated through action of Cdk1/2. In agreement with previous studies showing that both Mis18α and M18BP1 are putative targets of these kinases (Silva and Jansen, 2009; Silva et al., 2012, PhD thesis Mariana Silva, Universidade Nova de Lisboa, 2012), we now add that Mis18β centromere targeting operates through the same principle. In addition, we describe the identification a single residue (T653) within largest member of the Mis18 complex, M18BP1 that controls its timely centromere targeting. Based on our results we propose that temporal control over M18BP1 centromere localization is achieved through a direct phosphorylation of this residue by Cdk1/2, which occurs throughout interphase, resulting in M18BP1 sequestration from the centromere (Fig 2.2 and Stankovic et al., 2017). Upon mitotic exit, this residue is dephosphorylated allowing M18BP1 centromere targeting (Figure 2.3). Therefore negative signals derived from Cdk1/2 activities, together with positive phosphorylation signals driven by Plk1 activity allow for a cell cycle dependent behavior of M18BP1 (McKinley and Cheeseman, 2014; Silva et al., 2012; Stankovic et al 2017).

Given that Plk1 phosphorylation of its substrates often relies on a licensing phosphorylation driven by Cdks (Elia et al., 2003; Zhang et al., 2009), in the future, it would be interesting to determine whether phosphorylation of the T702 residue (a substrate for Plk1) depends on the phosphorylation of the T653 residue (a substrate for Cdk1/2) of M18BP1. Even though we have successfully uncoupled M18BP1 centromere localization from cell cycle control, we observed no detectable premature CENP-A deposition (in the case of transient transfection of GFP M18BP1^{T653A}). Upon constitutive

centromere targeting of this M18BP1 mutant, a minute but detectable level of CENP-A was preciously loaded at G2 centromeres. Hence, we conclude that other factors besides M18BP1 are contributing to the cell cycle dependent deposition of CENP-A.

Furthermore, we show that phosphorylation of T653 does not determine M18BP1 interaction with Mis18α in G2 phase, indicating that in G2 phase, phosphorylation of M18BP1 doesn't prevent interaction with Mis18α. However, this results is inconsistent with findings of (McKinley and Cheeseman, 2014) who reported the lack of co-recruitment of Mis18α upon expression of translation fusion between C-terminal domain of CENP-C and M18BP1, which allowed constitutive centromere targeting of this protein fusion throughout interphase. Expression of wild type CENP-C-M18BP1 did not co-recruit Mis18α in any stage of interphase and mitosis. However, expression of its counterpart in which 18 putative Cdk phospho-sites are mutated to Alanine resulted in mitotic Mis18α recruitment. One possible explanation for these discrepancies could be the differences in the amount of M18BP1 protein that is artificially placed at G2 centromeres due to different tethering strategies. In the case of the CENP-B tether, due to large array of available sites for its binding at α-satellites, Cbdbd-M18BP1 fusion is likely to be present in higher amounts compared to CENP-C-M18BP1 fusion. The fact that we can detect Mis18α co-recruitment under this condition indicates that it either does not exists or that Cdk1/2 inhibition can be overridden, implying that at least in G2 phase, Cdk1/2 do not play a major role in regulating the Mis18 complex formation. Additionally, forced recruitment of phosho-mimetic Cbdbd-M18BP1^{T653D} resulted in a similar co-recruitment level of Mis18α, arguing that T653 doesn't participate in the regulation of Mis18α and M18BP1 regulation. However, the involvement of other putative Cdk residues in this process cannot be excluded. This is

exemplified by the absence of Mis18α co-recruitment in mitotic stages. It is possible that in this cell cycle stage, when Cdk1 activity is at its highest levels, other conserved residues (e.g. T4, T40 or S110) are phosphorylated, resulting in inhibition of the interaction between M18BP1 and Mis18α (Pan et al., 2017).

The fact that we do not detect co-recruitment of Mis18β at G2 centromere upon tethering of M18BP1 is in agreement with direct binding of Mis18β to CENP-C at the centromere, rather than M18BP1 (Stellfox et al., 2016). This study proposed that Mis18β, similarly to Eic1 (Subramanian et al., 2014) and Mis19 (Hayashi et al., 2014), bridges Mis18α and M18BP1 complex to the centromeres by providing an additional binding surface to CENP-C via the Mis18β YIPPEE domain. Binding of both M18BP1 and Mis18β to CENP-C occurs through the recognition of the C-terminal tail of CENP-C, raising the possibility that M18BP1 and Mis18β are competing for their centromere occupancy with each other (Shono et al., 2015; Stellfox et al., 2016). This model is consistent with the observation that increasing the centromere residence time of M18BP1 in G1 phase, impacts the efficiency of Mis18β targeting, but not Mis18 alpha (Figure 2.10). Although highly speculative, I would like to propose that M18BP1 and Mis18α form a stable complex at the centromere, whose primary function is to attract histone-acetyltransferases, which would in turn form a permissive chromatin environment for nascent CENP-A deposition. Centromere localization of M18BP1-Mis18α would occur upstream of Mis18β and HJURP. Following M18BP1-Mis18α centromere targeting, centromeric chromatin is primed for nascent CENP-A deposition. CENP-A is deposited by its chaperone HJURP, which, as I will discuss in the chapter 3 of this thesis, is dephosphorylated upon mitotic exit, rendering HJURP active in CENP-A deposition. It has been suggested that the interaction between HJURP and

Mis18β is cell cycle regulated, though an inhibitory Cdk1-dependent phosphorylation of HJURP (Wang et al., 2014). Given the fact that we observe premature centromeric recruitment of M18β concomitantly with unscheduled loading of CENP-A (driven by expression of HJURP mutant), it is plausible that the centromeric targeting of Mis18β depends on its interaction with HJURP. Since we show that Mis18β centromere recruitment is Cdk regulated (Fig 2.8), but failed to identify any residue within M18β sufficient to alleviate this inhibition (PhD thesis Mariana Silva, Universidade Nova de Lisboa, 2012), it is quite likely that HJURP phosphorylation status is the key molecular determinant of the Mis18β and HJURP interaction, restricting Mis18β centromere localization to G1 phase. Therefore, it is probable that the expression of the $HJURP^{AxA}$ mutant (harboring decreased phosphorylation on its Cdk-responsive residues), is allowing Mis18β centromere recognition, which is, under these circumstances, occurring prematurely in G2 phase. Centromeric targeting of Mis18β-HJURP complex could rely on the interaction between centromeric Mis18α and Mis18β complexed with HJURP. Following initial centromeric recognition, an additional binding surface for Mis18β-HJURP complex is obtained through M18BP1 centromere displacement that is directly driven by competition for CENP-C binding between M18BP1 and Mis18β. This hypothesis is consistent with the observation that an increased residence time of M18BP1 at G1 centromeres negatively correlates with the efficiency of M18β centromeric recruitment.

In sum, I am proposing that the licensing of centromeric chromatin driven by the Mis18 complex is occurring in two discrete and co-dependent steps. The first one would involve targeting of the M18BP1-Mis18α complex that, via HATs, ensure the receptiveness of centromeric chromatin for nascent histone deposition. Centromeric targeting of M18BP1-Mis18α complex is

negatively regulated by Cdk1/2 activities. Upon mitotic exit, concomitantly with a drop in Cdk activities, M18BP1-Mis18α complex is efficiently targeted to centromeres, where they commence the process of chromatin priming for CENP-A deposition. The rapid accumulation of M18BP1-Mis18α at G1 centromeres is likely facilitated by the presence of low levels of M18BP1 at mitotic centromeres. The mitotic pool of centromere bound M18BP1 could serve as a platform for efficient recruitment of M18BP1-Mis18α complex in G1 phase, making this complex the most upstream factor regulating CENP-A deposition. Concomitantly with centromeric targeting of M18BP1-Mis18α complex, HJURP is dephosphorylated, rendering it active in the process of CENP-A deposition (See also chapter 3 of this thesis). Active HJURP recognizes and binds Mis18β, which in turns recruits HJURP to centromeres through interaction with centromere localized M18BP1-Mis18α complex. Initial binding of Mis18β-HJURP to Mis18α causes displacement of M18BP1 from the centromeres, thus freeing CENP-C tail for Mis18β binding. The interaction between Mis18alpha and M18beta, together with the one with CENP-C could contribute to the positioning of nascent CENP-A molecules adjacent to parental ones, as described previously (Ross et al., 2016).

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CHAPTER 3

Cdk1/2 dependent regulation of HJURP

This chapter contains sections of the publication: Ana Stankovic, Lucie Y. Guo, João F. Mata, Dani L. Bodor, Xing-Jun Cao, Aaron O. Bailey, Jeffrey Shabanowitz, Donald F. Hunt, Benjamin A. Garcia, Ben E.Black, Lars E.T. Jansen. A Dual Inhibitory Mechanism Sufficient to Maintain Cell-Cycle-Restricted CENP-A Assembly, *Molecular Cell*, 65.2 (2017), 231–46

Abstract

The centromere is responsible for chromosome segregation during mitosis. The chromosomal position of centromeres is epigenetically defined by nucleosomes containing the histone H3 variant CENP-A. In order not to lose this epigenetic mark, faithful CENP-A inheritance and propagation needs to be ensured. During S-phase, while DNA is replicated, CENP-A nucleosomes are semi-conservatively inherited and redistributed between the two sister chromatids. In contrast to canonical histones, CENP-A is not replenished during this phase which is delayed until after mitotic exit in early G1 phase. Previous studies from our laboratory showed that Cyclin-dependent kinases (Cdk) are the principal molecular triggers that prevent centromere propagation during S, G2 and mitotic phases and restrict centromere propagation to G1 phase of the cell cycle. Here we report further insight into the molecular mechanism underlying Cdk-based control of centromere inheritance. We have mapped a putative cyclin interaction site within the CENP-A specific chaperone HJURP which mediates Cdk1/2-dependent inhibitory phosphorylation of this protein. Alleviation of either cyclin A binding interphase or critical phospho-residues within HJURP is sufficient to alleviate cell cycle control over CENP-A deposition. Importantly, we demonstrate that negative control over CENP-A chaperone is exerted at the level of its centromere localization, rather than chaperoning capacity.

Introduction

3.1 Histone chaperones

Histones are highly basic and positively charged proteins, a property which mediates their high affinity towards negatively charged DNA. They are

involved in organizing eukaryotic DNA at the level of the fundamental unit of chromatin, the nucleosome (Kornberg, 1974). Given this intrinsic feature common for all histones, their unregulated production and accumulation in the cells can lead to deleterious effects through promiscuous interactions and aggregate formation. Thus, under most circumstances, when histones are not associated with DNA, they are bound to their respective histone chaperones (De Koning et al., 2007). These escort proteins prevent inadvertent interactions of histones with other factors, in part by neutralizing their charge, and also provide means to control histone variant supply and incorporation into chromatin. By definition, histone chaperones are the factors that associate with histones and stimulate a histone transfer reaction, without being a part of the final product (Burgess and Zhang, 2013; Laskey et al., 1978). Notably, not all histone chaperones mediate the actual deposition of histones onto DNA in vivo, and can play other important roles in histone dynamics, such is transfer of histones from one chaperone to another (a 'hand-off') (Liu and Churchill, 2012). Some histone chaperones, such is Asf1 (antisilencing function 1) bind and transfer histones without necessarily involving additional partners (De Koning et al., 2007), others, like CAF-1 (chromatin assembly factor-1) form complexes consisting of several chaperone units (Kaufman et al., 1995; Smith and Stillman, 1989; Zhang et al., 2016). Finally, histone chaperones can partner with factors which bear histone binding capacity within large enzymatic complexes, for example, Arp4 (actin-related protein-4) in the INO80 chromatin-remodeling complex (Kapoor et al., 2013; Morrison and Shen, 2009). Of particular interest is the human RbAp48 (retinoblastoma-associated protein 48) which also associates with the prenucleosomal CENP-A complex (Dunleavy et al., 2009). This chaperone displays high plasticity in terms of its binding partners which is context-dependent.

RbAp48 can function independently, yet it is also part of the three subunits of the CAF-1 complex, and of several histone remodeling and histone modifying enzymatic complexes (Gurard-Levin et al., 2014). The putative role of RbAp48 along with RbAp46 in propagation of CENP-A containing nucleosomes is discussed in chapter 2 of this thesis.

In addition to binding to histones, these chaperones also associate with other kay factors which form an interface between histone/chromatin dynamics and DNA metabolism. These interactions mediate temporal and spatial control over histone deposition. For example, during DNA replication or repair, CAF-1 is targeted to sites of DNA synthesis through its interaction with PCNA (proliferating cell nuclear antigen) (Moggs et al., 2000). During transcription, Spt6 (H3-H4 histone chaperone involved in transcription elongation) associates with the transcriptional machinery through interaction with RNA polymerase (Yoh et al., 2007). Likewise, the CENP-A chaperone HJURP, recognizes subunits of the Mis18 complex, which allows centromere-specific incorporation of nascent CENP-A (Dunleavy et al., 2009; Foltz et al., 2009; Nardi et al., 2016; Perpelescu et al., 2015; Wang et al., 2014). In sum, association of histones with cognate chaperones and their binding partners ensure that histones are incorporated into chromatin at the right time and at the right place when and where they are needed.

3.2 Assembly of CENP-A nucleosomes is mediated by a dedicated chaperone

In order for centromeric chromatin to be faithfully propagated, factors involved in CENP-A assembly have to have a capacity to first, recognize chromatin bound, centromere-localized CENP-A nucleosomes, and, secondly, to incorporate newly synthetized CENP-A molecules onto pre-

existing CENP-A nucleosome “templates” to compensate for the replicative dilution of nucleosomes during DNA replication. The remarkable stability of CENP-A nucleosome is discussed in the chapter 1 of this thesis. While there are few factors identified thus far to be involved in maintenance of CENP-A along the cell cycle, a great deal has been learned about the process of centromere assembly (Barnhart et al., 2011; Bassett et al., 2012a; Dunleavy et al., 2009; Foltz et al., 2009; Guse et al., 2011; Moree et al., 2011; Silva et al., 2012). A key player in this process is the CENP-A assembly factor HJURP, which has been found in tetrapods as well as a choanoflagellate (Barnhart et al., 2011; Dunleavy et al., 2009; Foltz et al., 2009; Sanchez-Pulido et al., 2009). A homolog, called Scm3, is also present in fungi (Camahort et al., 2007; Mizuguchi et al., 2007; Pidoux et al., 2009; Stoler et al., 2007; Williams et al., 2009).

Initial experiments using the genetically defined centromeres of budding yeast, identified Scm3 (suppressor or chromosome misalignment) as a non-histone protein interacting with both soluble and chromatin bound fractions of Cse4 (Camahort et al., 2007; Mizuguchi et al., 2007; Stoler et al., 2007). Its centromeric localization together with direct association with Cse4 placed Scm3 as one of the most upstream factors regulating kinetochore assembly, given that conditional inactivation of Scm3 results in chromosome missegregation and kinetochore disassembly. In addition, the N-terminal region of Scm3 was found to be responsible for recognition of the histone-fold-domain (HFD) of Cse4, a feature that remained remarkably conserved across different species (Bassett et al., 2012b; Cho and Harrison, 2011; Shuaib et al., 2010; Stoler et al., 2007). Replacement of the HFD of Cse4 with the corresponding one of H3 abolished the interaction with Scm3 (Stoler et al., 2007). (Mizuguchi et al., 2007) reported the formation of hexameric centromeric nucleosomes containing

$(Cse4/H4/Scm3)_2$, casting a doubt on whether Scm3 is indeed performing a chaperoning function for Cse4. Additionally, targeting of Scm3 to non-centromeric loci using an inducible Gal4 DNA binding domain was not sufficient to initiate ectopic Cse4 chromatin incorporation, which raised a possibilities that either this construct is not functional or that the input of underlying DNA sequence is vital for establishments of centromeric chromatin (Camahort et al., 2007). However, (Shivaraju et al., 2011) clearly demonstrated that Scm3 preferentially assembles Cse4 octamers *in vitro*, irrespective of the DNA template sequence. Purification and crystallization of Scm3 from *Kluyveromyces lactis* showed that the conserved N-terminal portion of Scm3 forms extensive interactions with the α2 helix of Cse4, while simultaneously capping the C terminal portion of the α2 helix preventing its association with DNA (Cho and Harrison, 2011). Importantly, association between the Cse4/H4 dimer and Scm3 prevents tetramer formation, strongly arguing against hexameric nucleosome composition $(Cse4/H4/Scm3)_2$ (Mizuguchi et al., 2007). Furthermore, *in vitro* nucleosome reconstitution assays showed that whereas Smc3 does assemble octameric Cse4 nucleosomes, it is not the part of the final nucleosomal structure (Dechassa et al., 2011).

Following the initial characterization in *S. cerevisiae*, a homologue of Scm3 was identified in fission yeast (Scm3sp) (Pidoux et al., 2009; Williams et al., 2009). Both of these studies established a causal link between centromere localization of Scm3 and the CENP-A licensing factor, the Mis16/Mis18 complex. In addition, they demonstrated a cell cycle dependent centromeric localization of Scm3. In contrast to Cnp1 which constitutively resides at the centromere, Scm3sp is displaced from it at the onset of mitosis, and re-associated following sister chromatid separation. Stable association of soluble Scm3 and Cnp1, along with the loss of centromeric Cnp1 in its

absence, and the transient targeting of Scm3 to the centromere, are all characteristics which suggest a role for Scm3 as a genuine Cnp1 histone chaperone.

Studies in human cells identified HJURP as a binding partner of soluble, prenucleosomal CENP-A. HJURP is targeted to centromeres in early G1, concomitant with the onset of CENP-A deposition (Dunleavy et al., 2009; Foltz et al., 2009; Shuaib et al., 2010). HJURP depletion results in abrogation of efficient CENP-A deposition, leading to a high frequency of mitotic errors. Importantly, HJURP discriminates between CENP-A and H3 molecules, and preferentially assembles CENP-A nucleosomes *in vitro* (Bassett et al., 2012). Artificial targeting of HJURP to an ectopic array is sufficient to induce the formation of a stable and heritable centromere in vivo (Barnhart et al., 2011). Together, these observations identify HJURP as a *bona fide* centromeric CENP-A histone assembly factor.

Surprisingly, homologs of Scm3 or HJURP have not been identified in *C. elegans* or plants, whereas in Drosophila, a protein, CAL-1 (chromosome alignement defect 1) containing a ´´Scm3-domain´´-like region was isolated as a CID specific chaperone (Chen et al., 2014; Erhardt et al., 2008; Schittenhelm et al., 2010). CAL-1 is found only in the *Diptera* genus (Phansalkar et al., 2012), is essential for CENP-A localization while being recruited to centromeres at a similar time as CENPA, and interacts with CENP-A in both chromatin and pre-nucleosomal complexes. Until recently, in *C.elegans*, M18BP1^{KNL2} was the only factor known to be required for CENP-A^{HCP-3} centromeric localization (Maddox et al., 2007). A recent study identified RbAp46/48^{Lin-53} complex to be required for deposition of CENP-A^{HCP-3} (Lee et al., 2016). In contrast to its role at fission yeast and human centromeres, in *C.elegans* embryos, RbAp46/48 is not modulating the

levels of acetylation of histones H3 or H4; its sole role appears to be escorting CENP-A^{HCP-3} to holocentric centromeres in M18BP1^{KNL2}-dependent manner.

3.3 Molecular basis of HJURP CENP-A selectivity

Both HJURP and Scm3 contain a short region with significant sequence conservation, the Scm3 domain (Cho and Harrison, 2011; Hu et al., 2011; Zhou et al., 2011), which forms an extended (26 amino acid long) α helix that recognizes the α2 helix of the respective CENP-A homologue. In the case of humans, a domain of CENP-A comprised of a portion of loop 1 and α2 helix termed CATD (centromere targeting domain) serves as a recognition platform for the N-terminal portion of HJURP (Scm3 domain) (Hu et al., 2011). Within the CATD, the Scm3 domain of HJURP recognizes six exposed residues which are sufficient to confer its binding selectivity for CENP-A over H3 tetramers (Bassett et al., 2012). Importantly, disruption of the tetramerization interface in the α2 helix of CENP-A does not interfere with the HJURP recognition, yet these dimers fail to be stably incorporated into chromatin (Bassett et al., 2012). Binding of HJURP to soluble CENP-A/H4 dimers precludes formation of CENP-A/H4 tetramers, indicating that CENP-A, H4 and HJURP form a trimeric complex (Zhou et al., 2011) (Figure 3.1). Although the CATD is the minimal portion of CENP-A required for HJURP binding, crystal structure models of the (CENP-A/H4)2-HJURP complex revealed that in addition to contacting the α2 helix of CENP-A, HJURP β-sheets associate with α1 helix as well. This binding prevents spontaneous unfolding of α1 helix of CENP-A, which in turn provides additional stability to many of the α helices of the histone folds of CENP-A and H4, resulting in increased rigidity of the overall $(CENP\text{-}A/H4)_2$ complex. Therefore, HJURP serves not only as an escort chaperone for CENP-A,

additionally it stabilizes the pre-nucleosomal CENP-A complex (Bassett et al., 2012a; Hu et al., 2011). Interestingly, the conserved Scm3 domain is highly similar to another histone chaperone DAXX, which recognizes yet another H3 variant H3.3 (Elsässer et al., 2012). The N-terminal 'DxxLxxRL' motif in facts mimics the thermodynamically favored inter-histone interactions which are formed amongst α3 helixes in the core of octameric nucleosome (Elsässer, 2013). Therefore, a common structural theme within this domain allows for recognition of histone surfaces in general, however subtle amino acid changes (amino acid X in 'DxxLxxRL' stretch) specific for each chaperone confer their specificity for respective histone variants. A prime example for this is the co-evolution of CID loop1 with the corresponding N terminus of CAL-1 within Diptera lineage in which rapidly evolving CID dictates fixation of compatible amino acid residues in its chaperone, resulting in a species-specific compatibility between a histone variant and its chaperone (Rosin and Mellone, 2016).

The composition of CENP-A nucleosomes has been the subject of intense investigation and debate. The extensive body of evidence (including X ray crystallography data, biochemical and mutational analyses) points to an octameric CENP-A nucleosome $(CENP\text{-}A/H4/H2A/H2B)_2$, analogous to their histone H3-containing counterparts (Bassett et al., 2012; Tachiwana et al., 2011). Alternatively, AFM data and nucleosome crosslinking assays suggest the existence of tetrameric, ´´hemisomal´´ CENP-A/H4/H2A/H2B nucleosomes, at least during part of the cell cycle (Bui et al., 2012; Dalal et al., 2007). Despite these unconventional nucleosome proposals, single-molecule fluorescence measurements of CENP-A nucleosomes and high-resolution DNA protection assays of centromeric chromatin, indicated that octamers represent the most prevalent form of CENP-A nucleosomes *in vivo* (Hasson et al., 2013; Paeganeh et al., 2013). In order to achieve an

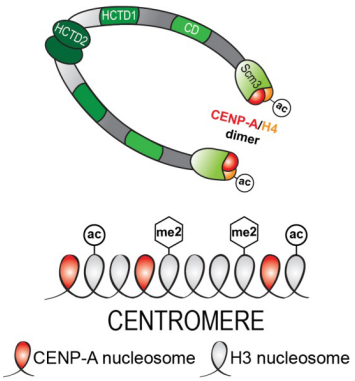
octameric composition of CENP-A nucleosome, containing a tetrameric $(CENP\text{-}A/H4)_2$ complex, HJURP dimerizes through its C-terminally located domain termed HCTD2 (HJURP C-terminal domain 2) (Zasadzińska et al., 2013) (Figure 3.1). Dimerization is necessary for chromatin deposition of nascent CENP-A, whereas an adjacent domain – HCTD1 (HJURP C-terminal domain 1) is required for centromere targeting of HJURP and was proposed to be a binding surface for members of the Mis18 complex (Nardi et al., 2016; Wang et al., 2014; Zasadzińska et al., 2013).

3.4 Additional factors involved in the efficient HJURP/CAL-1-mediated CENP-A deposition

Similarly to its fission yeast counterpart, HJURP centromeric targeting is dependent on its interaction with the members of the Mis18 complex (Barnhart et al., 2011; Perpelescu et al., 2015). In human cells, Mis18β recruits HJURP to G1 centromeres, while in chicken DT40 cells this role if performed by M18BP1 (Perpelescu et al., 2015). Additionally, a permissive chromatin environment generated by the combinatorial action of histone acetyltransferases and methyltransferases plays a pivotal role in the efficient centromeric targeting of HJURP (Figure 3.1). Both of the chromatin modifications, H3K9ac and H3K4me2 respectively, are associated with active transcription, suggesting that transcriptional activity of centromeric DNA contributes to maintenance of an active centromere (Bergmann et al., 2011; Ohzeki et al., 2012, 2016). Indeed, human α-satellites are transcribed during mitosis and early G1 phases, generating a long non-coding RNA, which partners with pre-assembled CENP-A complex. Binding of this type of RNA to soluble HJURP has been proposed to play a role in the CENP-A assembly process (Quénet and Dalal, 2014). Similarly, CAL-1 mediated loading of Drosophila CENP-A homologue-CID at the ectopic genomic

locus, is also dependent on local transcription driven by the FACT (facilitates chromatin transcription) complex (Chen et al., 2015) (Figure 3.1). Furthermore, transcription of Drosophila´s X chromosome gives rise to a long noncoding RNA-SATIII, which binds to the kinetochore protein CENP-C and influences the level of CID at all chromosomes (Rošić et al., 2014). While forming a complex with soluble CENP-A, HJURP also contacts the RbAp46/48-HAT1 complex, which in turn acetylates lysine 5 and 12 of H4 within the CENP-A prenucleosomal complex, highlighting that the general principles of chromatin assembly are utilized in the case of centromeric chromatin assembly as well (Shang et al., 2016) (Figure 3.1). Recently, an unexpected factor, diaphanous formin mDia2, has been implicated in the regulation of centromeric CENP-A levels (Liu and Mao, 2016). Depletion of mDia2 increases the residence time of HJURP at G1 centromeres, however, efficient deposition of nascent CENP-A is halted, probably due to the inactivation of the MgcRacGAP-dependent pathway of CENP-A ´´maturation´´. Interestingly, MgcRacGAP has been proposed previously to act as a ´´stabilizing´´ factor for nascent CENP-A nucleosomes, meaning that it recognizes newly incorporated chromatin-bound CENP-A and prevents it from being lost (Lagana et al., 2010) (Figure 3.1). This reaction was proposed to occur in mid G1 phase, following CENP-A deposition. However, depletion of mDia results in incapacity of HJURP to deposit newly-synthetized CENP-A, occurring in early G1 phase. These results may suggest that the stabilization of nascent CENP-A occurs simultaneously with its deposition, starting already in early G1 phase, and continuing throughout interphase. HJURP is not required for the maintenance of freshly deposited CENP-A nucleosomes (Bodor et al., 2013) and recruits assisting factors that contribute to efficient CENP-A deposition. One of these factors is Condensin II, which in Xenopus egg

extracts prevents eviction of nascent CENP-A nucleosomes (pointing to its role in CENP-A stabilization) (Bernad et al., 2011). On the other hand, Condensin II in human cells is co-recruited together with HJURP to G1 centromeres where is counteracts HJURP-driven chromatin decompaction; lack of Condesin II leads to a moderate loss of newly-loaded CENP-A (Barnhart-Dailey et al., 2017; Samoshkin et al., 2009) (Figure 3.1).



Additional factors involved in the efficient Scm3/HJURP/CAL-1-mediated CENP-A deposition	
Factor	**Publication**
The Mis18 complex	(Barnhart et al., 2011; Nardi et al., 2016; Pidoux et al., 2009; Stellfox et al., 2016; Williams et al., 2009)
H3K9ac	(Ohzeki et al., 2012, 2016)
H3K4me2	(Bergmann et al., 2011; Sullivan and Karpen, 2004)
Condensin II	(Barnhart-Dailey et al., 2017; Bernad et al., 2011)
FACT	(Chen et al., 2015)
RbAp46/48	(Shang et al., 2016)
Long non-coding RNA	(Quénet and Dalal, 2014; Rošić et al., 2014)
mDia2	(Liu and Mao, 2016)
CENP-B	(Fachinetti et al., 2015)
CENP-C	(Chen et al., 2014; Dambacher et al., 2012; Moree et al., 2011; Shono et al., 2015)
CENP-I	(Okada et al., 2006; Shono et al., 2015)
Modulo	(Chen et al., 2012; Padeken et al., 2013)

Figure 3.1 Left: Schematic representation of human HJURP protein along with the previously recognized functionally relevant domains: Scm3 domain (CENP-A binding), CD (conserved domain), HCTD1 (HJURP C-terminal Domain-1, responsible for HJURP centromere targeting) and HCTD2 (HJURP C-terminal Domain-2, mediates HJURP dimerization). Other key steps involved in HJURP/CENP-A centromere targeting are highlighted (acetylation of histone H4 within CENP-A/H4/HJURP trimeric complex) along with a combination of specific centromere histone marks. Right: Table summarizing factor identified thus far to be involved in Scm3/HJURP/CAL-1-driven CENP-A assembly.

Surprisingly, one publication put forward the idea that CENP-C, a member of CCAN network which is responsible not only for kinetochore nucleation (Guse et al., 2011), but also for CENP-A stabilization (Falk et al., 2015), is part of the soluble CENP-A/HJURP complex (Tachiwana et al., 2015). Moreover, centromeric localization of CENP-C had been proposed to depend on HJURP, suggesting that HJURP serves as a loading factor for

CENP-C as well. However, even though HJURP lacking the CENP-A binding Scm3-domain can, up to limited extent, interact and co-recruit C-terminal portion of CENP-C, it never did so in the case of full-length CENP-C. Moreover, dynamics of these two proteins at the centromeres do not reflect dependency of CENP-C on HJURP chaperoning activity as CENP-C is rapidly turned over in G1 and G2 phase (Hemmerich et al., 2008), whereas HJURP is targeted to centromeres in late telophase/early G1 phase. Therefore, further studies are necessary to solidify the putative interaction and the contribution of HJURP to CENP-C centromere localization.

3.5 Molecular mechanism of HJURP centromere targeting

In most animal systems examined, CENP-A displays a unique pattern of cell cycle-coupled replenishment, whereby assembly of newly synthesized CENP-A is delayed until mitotic exit, in G1 phase of the next cell cycle, after the primary function of the centromere has been fulfilled. This process is negatively regulated by Cdk1/2 activities; brief inhibition of these kinases is sufficient to drive CENP-A deposition prior to mitotic exit (Silva et al., 2012). This has led to a model where the CENP-A assembly machinery is present and poised for activity but is kept inactive throughout S, G2 and M phase, until mitotic exit when activities of Cdk1/2 drop, concomitant with the onset of CENP-A deposition. HJURP is a phosphoprotein (Dephoure et al., 2008; Kato et al., 2007) and, consistently with its chaperoning function, displays a dynamic localization pattern being transiently targeted to centromeres at late telophase/early G1 phase (Dunleavy et al., 2009; Foltz et al., 2009). Even though CENP-A itself is modified on multiple residues (Bailey et al., 2013, 2015), the fact that $H3^{CATD}$ chimera retains G1-resticted chromatin incorporation indicates that CENP-A loading factors are putative targets of

cell cycle-based regulation, rather than CENP-A itself (Bodor et al., 2013). These features render HJURP as one of the prime targets for Cdk-based regulation. Interestingly, outside of this brief cell cycle window when it is centromere localized, HJURP (together with prenucleosomal CENP-A) is stored in nucleoli (Dunleavy et al., 2009; Foltz et al., 2009; Stankovic et al., 2017). This feature will be further discussed later in this chapter. In addition, recent work has proposed that CENP-A serine 68 is phosphorylated by mitotic Cdk activity (Yu et al., 2015). However, mutation of this residue does not lead to a change in the timing of CENP-A deposition (Yu et al., 2015), and the functional importance of this residue has been disputed (Fachinetti et al., 2017). In contrast, it has been reported that mutations of phospho-residues in HJURP result in premature centromere recruitment of HJURP, consequently leading to precocious deposition of CENP-A (Müller et al., 2014). Moreover, phosphorylation of residues within the HCTD1 seemingly negatively influences association of HJURP and Mis18β, limiting its centromeric recruitment to G1 phase of the cell cycle (Wang et al., 2014).

3.6 Material and Methods

DNA constructs

HJURP-GFP (pLJ381) and GFP-HJURP-ΔCLacI (pLJ632) (Zasadzińska et al., 2013), in which amino acids 483-743 (C-terminus) were replaced by dimerization domain of LacI (Zasadzińska et al., 2013) were a gift from Dan Foltz (Northwestern University). GFP-HJURP^{CDpoint} (pLJ696) mutant harbouring Alanine substitutions of residues P229, R230,D254, C256, N257, D262, L263, Y264 and M267 was cloned from a GeneString (Invitrogen) synthetic construct into pLJ381. GFP-HJURP^{CDdel} (pLJ704), in which residues spanning N231 through D253 were deleted was cloned from a GeneString (Invitrogen) synthetic construct into pLJ381. GFP-HJURP (pLJ380) and GFP-HJURP-ΔCLacI (pLJ632) were converted to GFP-HJURP^{AxA} (pLJ600) and GFP-HJURP^{AxA}-ΔCLacI (pLJ654) by quick exchange PCR replacing R276 and L278 by Alanine. GFP-HJURPS210A, S211A, S412A (pLJ828) or GFP-HJURPS210A, S211A, S412A-ΔCLacI (pLJ830) was made via quick exchange PCR. pLJ591, HJURP-CBdbd-GFP was created by PCR amplification of the first 158 N-terminal amino acids of CENP-B protein [CENP-B DNA binding domain (CBdbd)] and ligation to the N terminus of pLJ383 (a GFP-Mis18α construct)**.**

Cell lines

All human cell lines used were grown at 37°C, 5% CO2. Cells were grown in DMEM (Bio West) supplemented with 10% fetal bovine serum (FBS) (BioWest)), 2 mM glutamine, 1 mM sodium pyruvate (SP) (Thermo Fischer Scientific), 100 U/ml penicillin, and 100 µg/ml streptomycin, with the exception of HeLa HILO derived cell lines in which 10% tet-free (BioWest) FBS was used. HeLa HILO RMCE cell lines were a gift from E.V. Makeyev,

Nanyang Technological University, Singapore, and contain a single genomic recombination which allows for the insertion of a tetracycline responsive expression cassette (Khandelia et al., 2011). HeLa HILO RMCE clone #10 was transfected with 2,5 ng/µl of pLJ745 and pLJ746, vectors carrying two loxP sites flanking the Doxycycline (Dox) inducible 3xFlag-HJURP or 3xFlag-HJURP^{AxA} expression construct. Cre recombinase (Khandelia et al., 2011) was added at 1% of total DNA content. Positive clones were selected using 1 µg/ml of Puromycin (MERCK). Expression of 3xFlagHJURP/HJURP$^{WT/AxA}$ was induced by 10 µg/ml of Doxycycline (Sigma-Aldrich) and assayed for equal expression by western blot using FlagM2 antibody (Sigma-Aldrich). U2OS CENPA-SNAP cell lines were gift from Genevieve Almouzni (Institut Curie, France).

DNA transfection and siRNA treatment

Transient transfection of HeLa CENP-A-SNAP and HEK293T was performed using Lipofectamine LTX (Invitrogen; Carlsbad, CA) according to the manufacturer's instructions. All siRNAs were obtained from Dharmacon. HJURP was depleted as previously reported (Zasadzińska et al., 2013).

Co-Immunoprecipitation

HEK293T cells were transiently transfected either with GFP alone, GFP-HJURP-ΔCLacI or GFP-HJURP^{AxA}-ΔCLacI. 24h post-transfection cells were either allowed to continue to cycle or were treated overnight in DME III to induce mitotic arrest. 48h post-transfection, 107 cells were harvested in ice-cold PBS for 5 min at 3,200 x g and lysed in buffer containing 3,75mM Tris pH 7,5, 20mM KCl,0,5mM EDTA, 0,1% digitonin and 0,4 µM DTT. Lysates were homogenized using a 27G needle, and spun at 300 x g for 5 min. This was repeated two times, followed by combining two

supernatants and spin clarification at 10,000 x g for 15 min. Soluble fraction was collected and KCl concentration was adjusted to 150 mM. 5µg/ml of anti-Cyclin A coated agarose beads (Santa Cruz Biotechnology) or anti-Cyclin B (Santa Cruz Biotechnology) was equilibrated in lysis buffer prior to pulldown. After immunoprecipitation, beads were washed once in wash buffer A (20mM HEPES, 20Mm M KCl, 0,4m M DTT and 0,4mM EDTA) and two times in wash buffer B (wash buffer A with 225mM NaCl for Cyclin A pulldown, and 150mM NaCl for Cyclin B). Complexes bound to the beads were eluted using 2% SDS for 20 min, followed by immunoblotting with anti-Cyclin A (Santa Cruz) (Figure 3.4A) or anti-Cyclin B (Santa Cruz) (Figure 3.4C) and anti-GFP (Chromotek) antibodies. IRDye800CW-coupled anti-rat (Licor Biosciences) and DyLight680-coupled anti-rabbit (Rockland Immunochemicals, Gilbertsville, PA) secondary antibodies were used prior to detection on an Odyssey near-infrared scanner (Licor Biosciences, Lincoln, NE). Immunoblot signals were quantified using the Odyssey software (see also (Bodor et al., 2014)). GFP signal values were normalized to their respective Cyclin A signals and to corresponding GFP input values.

SILAC and affinity purification of prenucleosomal HJURP/CENP-A/H4 complex

SILAC labeling medium (MEM Eagle Joklik Modification) deficient in lysine and arginine was reconstituted according to manufacturer's instructions (Sigma-Aldrich), and supplemented with normal lysine and arginine (Sigma-Aldrich) for “light” medium, and 50 mg/ L 13C6,15N2-lysine and 50 mg/L 13C6, 15N4-arginine (Silantes) for “heavy” medium. Both media were supplemented with 10% dialyzed FBS (Gemini), GlutaMax (Gibco), 1 mM HEPES, 1% Pen/Strep, MEM non-essential amino acids (Gibco), and 120

mg/L proline to prevent arginine-to-proline conversion. Two parallel cultures of previously characterized HeLaS3 cells stably expressing localization and purification (LAP)-tagged CENP-A (Bailey et al., 2013) were cultured in spinner flasks for at least 6 cell doublings to allow full incorporation of the stable isotope-containing amino acids. Heavy isotope labeling efficiency of ~98% was confirmed by mass spectrometry after trypsin digestion of proteins extracted from heavy-labeled cells. To enrich for mitotic cells, both cultures were treated with 50 µM S-trityl-L-cysteine for 17 h. Subsequently, the "light" cells were treated with 100 µM R-Roscovitine (AdipoGen) for 30 min while the "heavy" cells were mock-treated with DMSO. Cell cycle status and HJURP phospho-status was monitored by immunoblotting for H3pS10 (Upstate) and an anti-HJURP antibody generated against a C-terminal fragment (1 µg/ml) (Bassett et al., 2012, Dev Cell), respectively. Cell pellets from 1.4 x 109 of "light" and "heavy" cells were combined in 1:1 ratio. Affinity purification of the prenucleosomal HJURP/CENP-A/H4 complex was performed as previously described (Bailey et al., 2013) except that protein elution was performed with 2% SDS and heating at 95°C.

Mass spectrometry and data analysis

Purified CENP-A and associated proteins were precipitated using pre-chilled acetone (4 X volume) followed by successive washing. Dried protein pellets were reconstituted with 0.1% RapiGest SF Surfactant (Waters) in 100 mM NH4HCO3, pH 8.0. Resuspended proteins were reduced using DTT, alkylated with iodoacetamide, and digested using trypsin. Since trypsin cleaves only after lysines and arginines, this ensures that every resulting peptide will contain at least one lysine or arginine, so that all heavy peptides are distinguishable from their corresponding light peptides by predictable mass differences. Rapidgest was removed by adding 0.5%

TFA and incubation for 30min at 37°C. The peptides were desalted with StageTips (Rappsilber et al., 2007), followed by phosphopeptide enrichment by TiO2 prior to analysis by Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific). The pFind search engine was used to search the UniProt human protein database to identify peptides (Wang et al., 2007). Quantification was done using extracted-ion chromatograms (XICs) of each light and heavy peptide pair, and L/H ratio represents the ratio of total area under each elution peak. Mass spectra of a representative non-phosphorylated HJURP peptide from the flow through in Figure 3.11 of samples from cells containing HJURP and HJURP^{AxA} had a retention time range of 28.75-29.89min, which includes all scans in both runs in which the peptide was detectable. Mass spectra of the phosphopeptide containing pS210/pS11, from the elution of the phospho-enrichment from cells containing HJURP and HJURP^{AxA} had a retention time range of 24.00-25.48min, which includes all scans from both runs in which the peptide was detectable.

Cell synchronization

Double Thymidine-based synchronization was performed as described (Bodor et al., 2012a). For Mitotic synchronization, 2,4 µM of EG5 inhibitor III Dimethylenastron-DMEIII (Calbiochem) was used for 24h. For synchronous mitotic exit, following DMEIII washout, HeLa and Hek293T were released for 5h and 7h, respectively. Nocodazole was used at 100ng/ml.

SNAP quench-chase-pulse labelling

Cell lines expressing CENP-A-SNAP were pulse labeled as previously described (Bodor et al., 2012).

Immunofluorescence

Procedures are essentially as described (Bodor et al., 2012a) (See also supplemental experimental procedures). To detect GFP-HJURP^{AxA}-ΔCLacI on G2 centromeres, HeLa CENP-A-SNAP cells transiently expressing the construct were pre-extracted for 5min prior to fixation. Cells were counterstained using anti-CENP-T (Barnhart et al., 2011) and anti-Aurora B (1:100; BD transduction laboratories). GFP-HJURP^{AxA}-ΔCLacI signal was amplified using GFP-Booster Atto488 (Chromotek).

Microscopy

Imaging was performed using a DeltaVision Core system (Applied Precision) inverted microscope (Olympus, IX-71), coupled to a Cascade2 EMCCD camera (Photometrics). Images (ranging from 512x512 to 1024x1024) were acquired at 1 x binning using a 100x oil objective (NA 1.40, UPlanSApo) with 0, 2 µm z sections.

3.7 Results

HJURP is phosphorylated in a cell cycle dependent manner

HJURP, the CENP-A specific chaperone, is a phospho-protein and features several putative Cdk sites (Figure 3.2 and (Bailey et al., 2016; Dephoure et al., 2008; Kato et al., 2007; Müller et al., 2014; Wang et al., 2014), making it a prime candidate for cell cycle control of CENP-A assembly. To quantitatively measure HJURP phosphorylation we used stable isotope labeling by amino acids in cell culture (SILAC) coupled to mass spectrometry. This allowed us, in an unbiased manner, to precisely determine which residues are phosphorylated under high Cdk conditions and how these respond to changes in Cdk activity. Cdk1 levels differ most dramatically between mitosis and G1 phase. For this reason, we specifically compared levels of phospho-peptides on the prenucleosomal GFP-CENP-A/HJURP complex between populations of mitotically arrested cells and cells that are released from mitotic arrest by Roscovitine-mediated Cdk inhibition. Normal timing and efficiency of CENP-A assembly is preserved under these conditions (Figure 3.3). We detected 6 phosphorylated residues corresponding to putative Cdk consensus sites within HJURP, all of which were dephosphorylated to varying degrees upon mitotic exit, ranging from 25-70% decrease relative to mitotic values (Figures 3.2C). Although three of these sites (S412, S448, S473) correspond to recently reported phospho-sites (Müller et al., 2014; Wang et al., 2014), our analysis shows that these are neither the sole nor the most responsive sites to inactivation of Cdks, at least in mitosis. In contrast, no change is observed at unphosphorylated peptides of HJURP (Figure 3.2C) nor at Cdk-consensus phospho-sites on the CENP-A N-terminal tail (Bailey et al., 2013) after forced mitotic exit (Figure 3.2D), indicating that protein

levels of CENP-A and HJURP remain unaffected and that HJURP is selectively dephosphorylated. Together, our findings from SILAC experiments led us to focus on HJURP in particular, and determine how its phosphoregulation is coupled to the control of the cell cycle timing of CENP-A chromatin assembly.

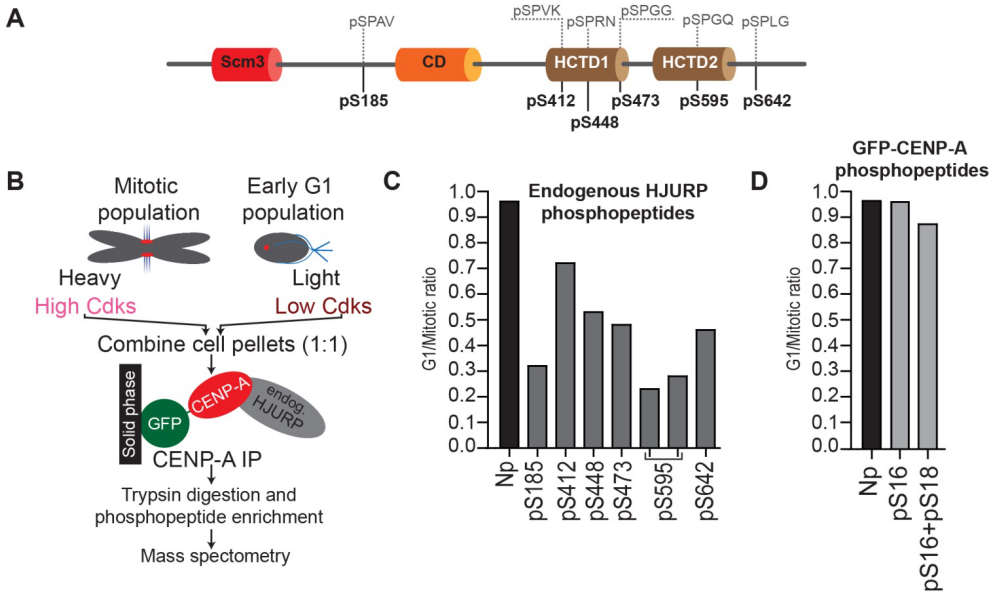


Figure 3.2 HJURP is phosphorylated in a Cdk dependent manner. Schematic representation of HJURP protein (Scm3: CENP-A binding domain; CD: Conserved Domain, HCTD (HJURP C-Terminal Domain). Position of phospho-sites identified by SILAC in C are indicated. Amino acid sequences flanking phospho-sites are annotated in grey. (A) Schematic of SILAC experiment. Light cells were released into G1 by Roscovitine treatment for 30 min. At this stage HJURP is partially dephosphorylated (Figure 3.3F, G). (C) The L/H ratios of phosphorylated Cdk sites detected on endogenous HJURP are listed. A representative non-phosphorylated peptide (Np) is shown as internal control. Note: pS595 was detected on two independent peptides. (D) L/H ratios of Cdk consensus sites within the N-terminal tail of CENP-A.

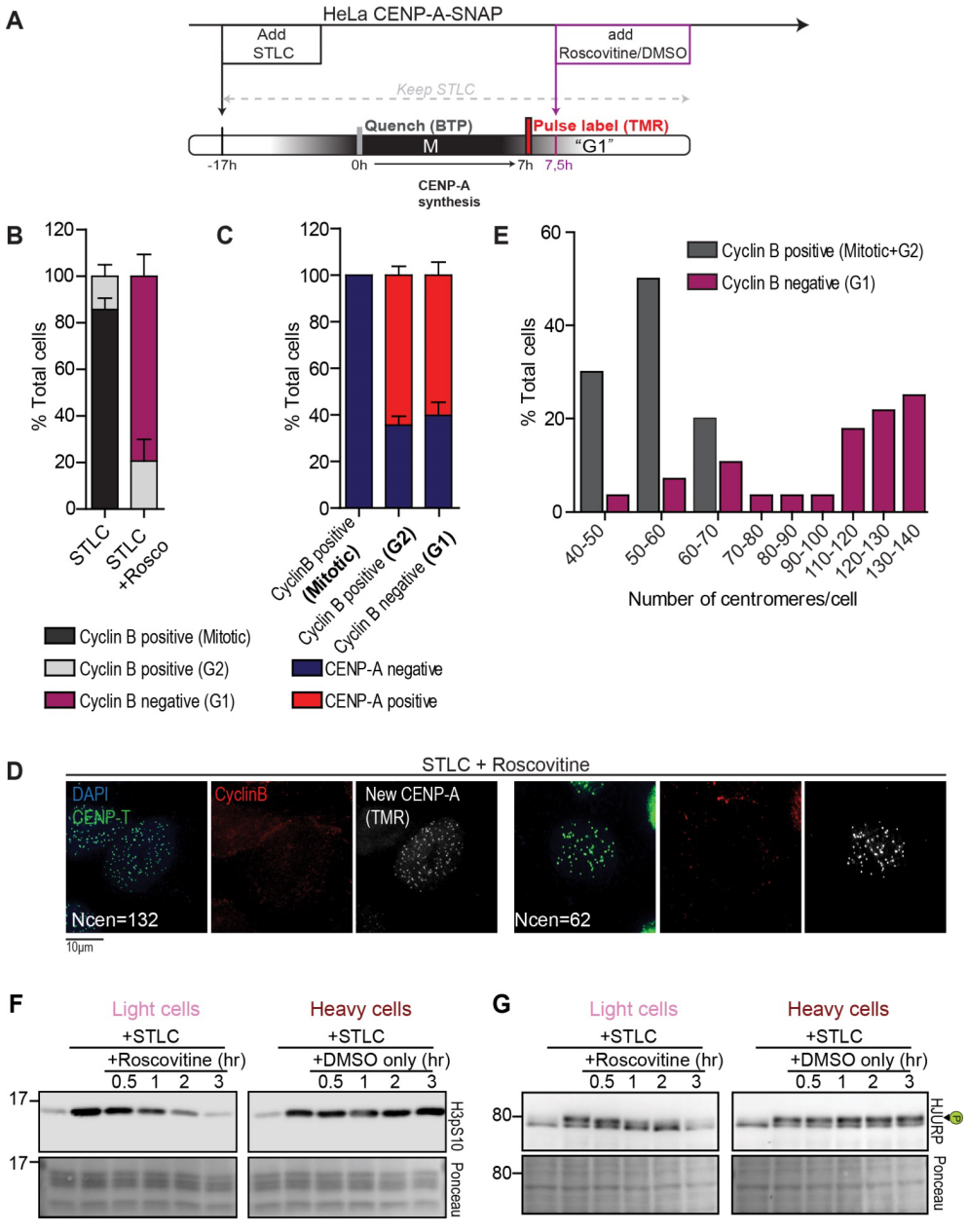


Figure 3.3 Conditions used for SILAC analysis are permissive for CENP-A deposition.
(A) To enrich for mitotic phase, HeLa CENP-A-SNAP cells were treated with Eg5 inhibitor (STLC) for 24h. 17h into mitotic arrest, pre-existing CENP-A-SNAP pool was quenched with

Figure 3.3 Continued: BTP, followed by 7h of chase. 30min before pulse labeling of newly synthesized pool of CENP-A-SNAP and fixation, cells were treated either with Roscovitine or DMSO. (B) Quantification of frequencies of Cyclin B positive (G2 and mitotic) cells and Cyclin B negative (tetraploid cells which exited mitosis without cytokinesis due to Cdk1 inhibition, see panel D, E) in the experiment described in (A). (C) Quantification of frequencies of CENP-A-SNAP (TMR) positive cells in each cell cycle stage present. Cells arrested in mitosis do not assemble CENP-A (left bar). Degree of CENPA assembly in either G2 or G1 cells is assayed following Roscovitine treatment. (D) Representative images of Roscovitine-treated cells in experiment described in (A). Cells were counterstained with Cyclin B, CENP-T and DAPI to indicate cell cycle status, centromeres and DNA respectively. (E) Distribution of number of centromeres under experimental conditions described in (A). Doubling of centromere number indicates formation of tetraploid cells due to forced mitotic exit in the presence of STLC and Roscovitine. (F) Western blots for the mitotic marker H3pS10 indicating cell cycle position of HeLa S3 cells used in SILAC experiment in Figure 3.2. Cells were arrested in mitosis with the Eg5 inhibitor STLC followed by treatment with DMSO control or Roscovitine (light cells) to force mitotic exit caused by Cdk inhibition. (G) Western blots for HJURP (isolated from soluble fraction) from HeLaS3 cells showing dephosphorylation (as seen by shift in SDS-PAGE mobility of phosphorylated HJURP) upon Roscovitine treatment of "light" cells. Based on this, we harvested cells after 30 min of Roscovitine (or DMSO) treatment, balancing between HJURP dephosphorylation and completion of HJURP-mediated centromeric chromatin assembly.

The HJURP conserved domain interacts with Cyclin A and controls timing of CENP-A assembly

Although the canonical consensus site for Cdks is (S/T)PX (K/R) (Hagopian et al., 2001; Holmes and Solomon, 1996), 5 of the 6 phospho-sites in HJURP that are affected by Cdk inactivation only display a shorter (S/T)P motif (Figure 3.2A) (Errico et al., 2010). Phosphorylation of such truncated motifs often requires additional cyclin binding sites for enhanced substrate recognition (Adams et al., 1996; Russo et al., 1996). Interestingly, we found a typical cyclin A binding RxL motif (Brown et al., 2007) within a vertebrate conserved domain (CD) of HJURP (Figure 3.4A), which has no previously

described function (Sanchez-Pulido et al., 2009). We tested whether HJURP indeed interacts with cyclin A and B, the major drivers of Cdk activity in S/G2 phase and mitosis, respectively, all stages at which CENP-A assembly is inhibited (Silva et al., 2012). In addition, we tested whether this putative interaction depends on the RxL motif. We performed either Cyclin B or Cyclin A co-immunoprecipitation using HEK293T cells in which we ectopically expressed either GFP-tagged HJURP with a mutated RxL motif (RLL>ALA, henceforth referred to as HJURP^{AxA}), or with a wild type conserved domain. In addition, to avoid cross-dimerization with endogenous HJURP, we replaced its homodimerization domain with that of LacI, which does not interfere with the CENP-A chaperoning and assembly activity of HJURP, as described previously (Zasadzińska et al., 2013) (henceforth named HJURP-ΔCLacI). Cyclin A robustly co-immunoprecipitated GFP-tagged HJURP-ΔCLacI in HEK293T cells (Figure 3.4A). In contrast, GFP-HJURP^{AxA}-ΔCLacI pulldown was reduced by 70% compared to HJURP-ΔCLacI, carrying a wild type conserved domain (Figure 3.4B). Mitotically enriched cells (low cyclin A) were used as a control to demonstrate that HJURP pulldown is cyclin A dependent. Consistent with the fact that inhibition of CENP-A assembly is maintained in mitosis (Jansen et al., 2007), even though cyclin A is degraded in early mitosis (den Elzen and Pines, 2001; Geley et al., 2001), we find that like cyclin A, cyclin B can interact with HJURP (Figure 3.4C). Interestingly, this interaction is not dependent on an intact conserved domain of HJURP, indicating inhibitory control in mitosis is exerted through a different mechanism.

Our mapping of the principal cyclin A interaction site on HJURP allowed us to determine the consequences of reduced cycling A binding to HJURP for the timing of its localization along the cell cycle. Upon removal of soluble

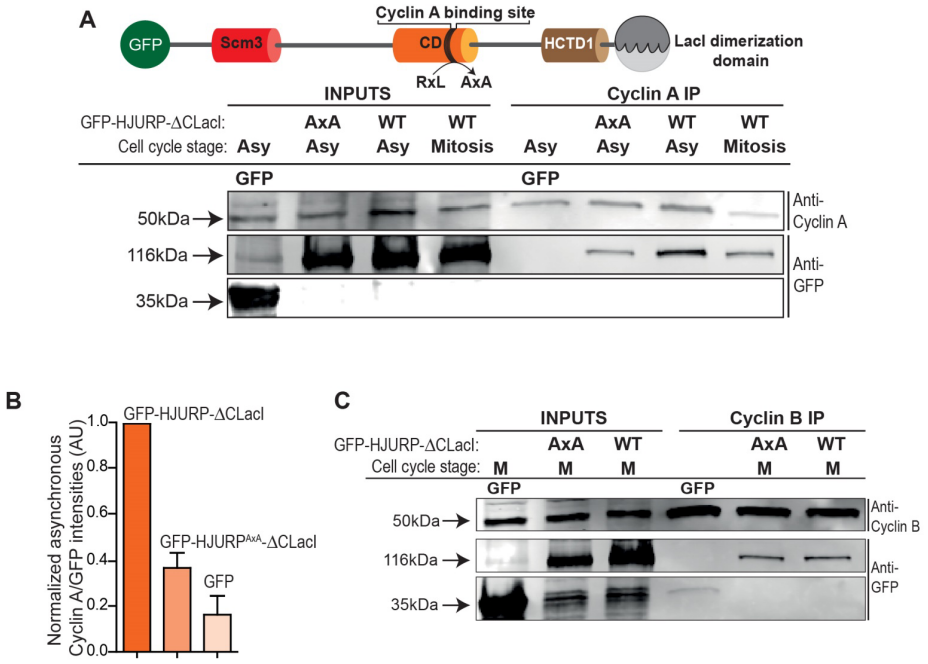


Figure 3.4. HJURP interacts with cyclin A and cyclin B. (A) HJURP CD mediates interaction with Cyclin A. (Top) Schematic representation of HJURP protein. Mutation of conserved RxL motif to AxA is annotated with black arrow. Experiments are performed with an HJURP construct in which the C-terminal homodimerization domain is replaced with that of LacI to prevent dimerization with wild type HJURP. (Bottom) co-IP of extracts expressing indicated constructs, either from asynchronous or mitotically enriched cells. Bound complexes were separated using SDS-PAGE followed by immunoblotting with indicated antibodies. (B) Quantification of IP experiments. GFP signal from each IP was normalized to corresponding cyclin A signal and input GFP signal in order to control for IP efficiency and GFP fusion protein expression level, respectively. GFP-HJURP signals were set to 1. Error bars indicate SEM (standard error of mean) from 3 independent experiments. (C) Cyclin B interacts with HJURP in mitosis independently of Conserved Domain. Randomly cycling HEK293T cells were transiently transfected either with GFP alone, GFPHJURP-ΔCLacI or GFP-HJURP^{AxA}-ΔCLacI. 24h post-transfection cells were treated overnight using DME III inhibitor to induce mitotic arrest. 48h post-transfection, cells were lysed and Cyclin B was immunoprecipitated using Anti-Cyclin B coated beads. Bound complexes were separated using SDS-PAGE followed by immunoblotting with indicated antibodies.

HJURP by pre-extraction we revealed the stably chromatin bound pool. While wildtype pre-mitotic HJURP is enriched in nucleoli [as observed previously (Dunleavy et al., 2009)], HJURP^{AxA} targeted to centromeres prematurely in G2 phase, the time of the cell cycle in which cyclin A is the principal cyclin (Figure 3.5).

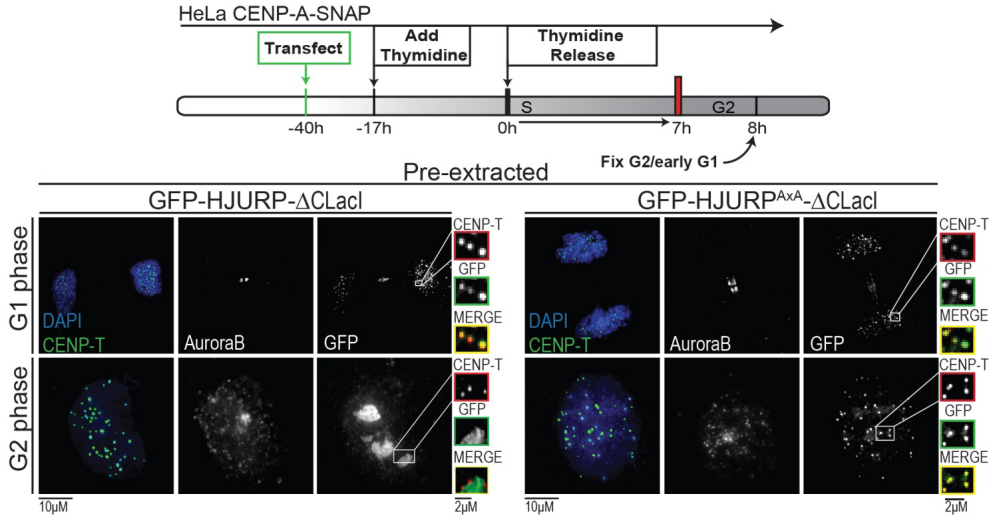


Figure 3.5 Timing of HJURP targeting is controlled by HJURP CD. HeLa CENP-A-SNAP cells were transiently transfected with indicated constructs and Thymidine synchronized to enrich cells in G2 phase. Cells were permeabilized prior to fixation and counterstained for Aurora B, CENP-T and DAPI to distinguish between G2 and early G1 cell cycle phases, centromere localization and DNA, respectively. GFP booster was used to amplify GFP-HJURP fluorescent signal.

In addition, we analyzed CENP-A deposition using a SNAP tag-based, fluorescent quench-chase-pulse labeling protocol that we described previously (Bodor et al., 2012; Silva et al., 2012). Remarkably, expression of the cyclin A binding mutant of HJURP resulted in a precocious deposition of nascent CENP-A in G2 phase. In contrast, expression of wild type GFP-HJURP never results in such pre-mitotic assembly (Figure 3.6).

Quantitative analysis showed that precocious CENP-A assembly occurred at the centromere and reached ~40% of G1 levels (Figure 3.6 A´´, B´) [20% assembly on a per centromere basis, when considering the replicated state of sister centromeres in G2 phase].

Interestingly, a small deletion or a series of point mutations of highly conserved residues within the HJURP conserved domain also resulted in premature deposition of CENP-A in G2 phase (Figure 3.7). These results suggest that an intact conserved domain is essential for a robust interaction with cyclin A beyond the RxL site and further solidify the primary role of this conserved domain as a cell cycle responsive element of HJURP. Given this, we wanted to test if a complete, in-frame-deletion of the full conserved domain (residues 228 to 304) would be sufficient to completely abolish the previously detected interaction with Cyclin A, since our HJURP^{AxA} mutant did so with ~70% efficiency (Figure 3.4B). However, deletion of the conserved domain resulted in expression of a truncated protein which size was far smaller than expected, preventing us from testing this hypothesis. Consistent with this observation, this mutant was not inducing premature CENP-A deposition, and moreover, it localized throughout the nucleus and cytoplasm, in sharp contrast to HJURP^{AxA} (data not shown). To determine whether HJURP^{AxA} itself is a functional assembly factor or whether it dimerizes with wild type copies of HJURP to achieve premature assembly, we replaced the essential C-terminal dimerization domain with that of LacI to prevent cross dimerization of HJURP^{AxA} with wildtype copies. Either GFP-HJURP^{AxA} or GFP- HJURP^{AxA}–ΔCLacI expression result in a similar level of precocious deposition of CENP-A (Figure 3.8A´, A´´). Moreover, downregulation of endogenous HJURP (Figure 3.8) showed no effect on either efficiency or frequency of premature CENP-A loading following GFP-HJURP^{AxA} expression (Figure 3.8 B´´, B´´´).

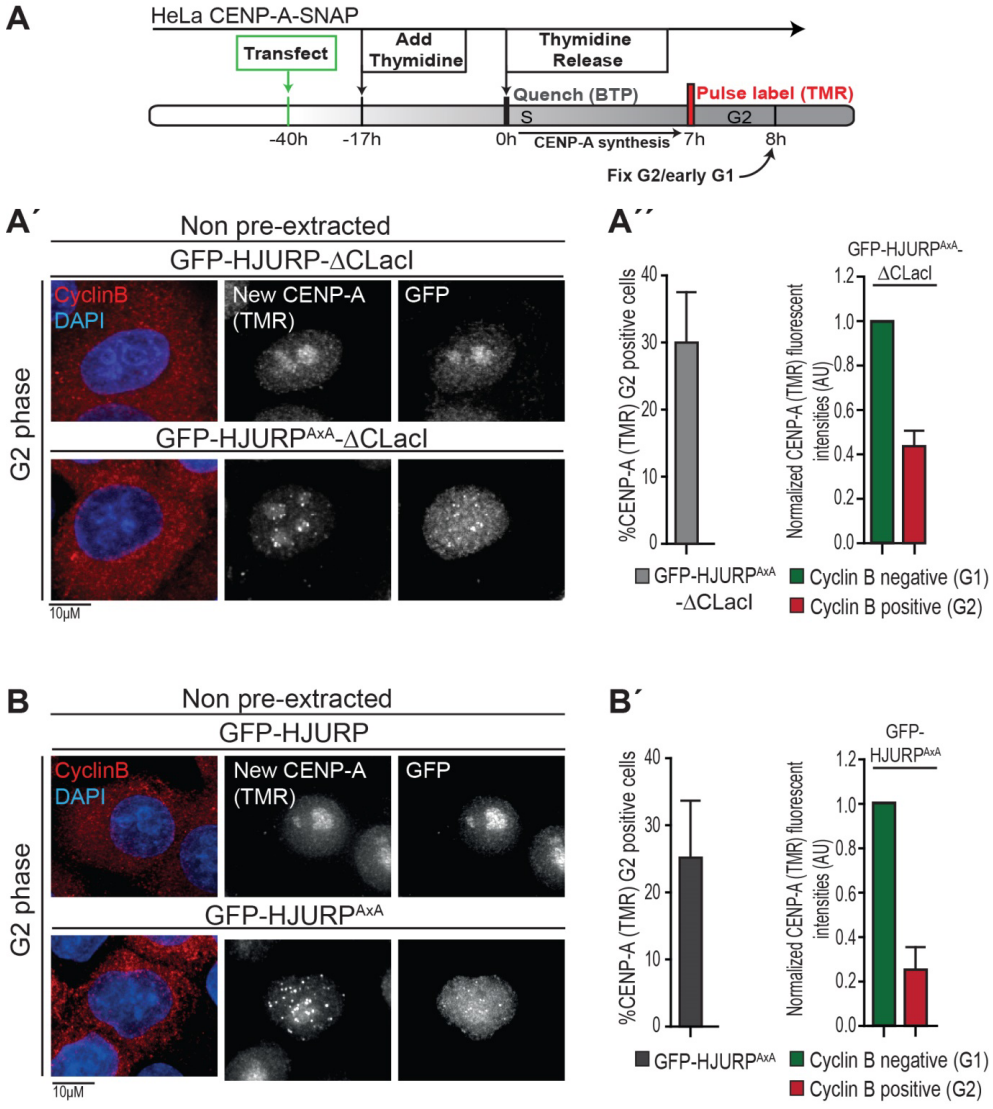


Figure 3.6. Timing of CENP-A assembly is controlled by HJURP CD. (A): Experiments were performed as in Figure 3.5 except here CENP-A assembly was assayed using SNAP TMR-labeling of its S phase synthesized pool. Following fixation, cells were counterstained for cyclin B and DAPI to indicate G2 status and DNA, respectively (A´). (A´´) Left: Quantification of frequency of premature CENP-A loading in Cyclin B positive cells

Figure 3.6 Continued: expressing GFP-HJURP^{AxA}-ΔCLacI. Right: Quantification of CENP-A-SNAP (TMR) fluorescent signal intensities of cells from experiment on the left in G2 phase (Cyclin B positive) and G1 phase (Cyclin B negative), using CENP-T signal as a centromere reference (not depicted). Centromeric CENP-A-SNAP fluorescent signals were normalized to average of G1 cells signals in each experiment (not considering the difference in replicated sister G2 centromeres vs. segregated G1 centromeres). 3 replicates, error bars indicate SEM. (B, B´) Experiments are identical to the one described in A, with exception of transfection with GFP-HJURP and GFP-HJURP^{AxA}. 3 replicates, error bars indicate SEM.

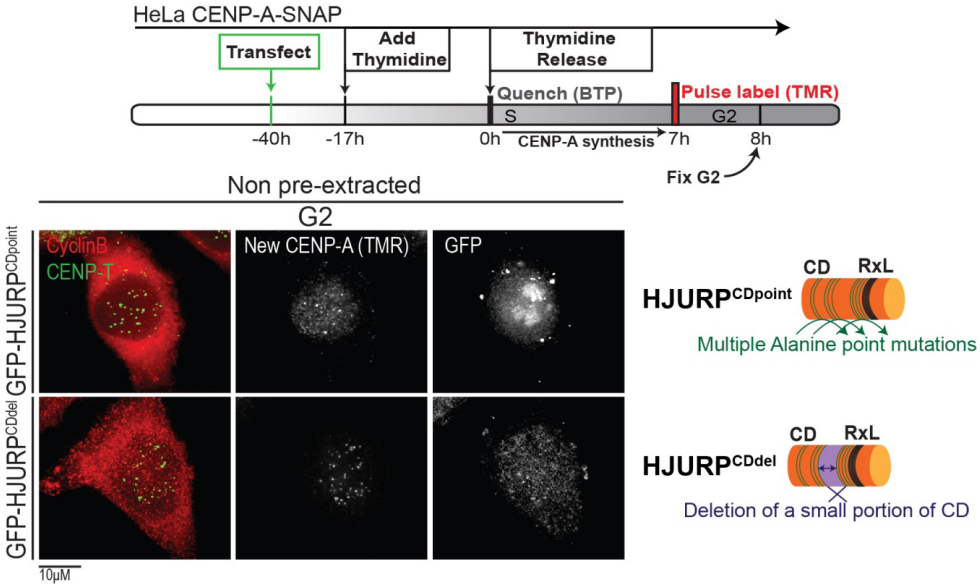


Figure 3.7 An intact conserved domain is necessary for maintenance of cell cycle control over CENP-A assembly. HeLa CENP-A-SNAP cells were transiently transfected with indicated constructs. CENP-A assembly was assayed using SNAP TMR-labeling of its S phase synthesized pool. Following fixation, cells were counterstained for cyclin B, DAPI or CENP-T to indicate G2 status, DNA and centromeres respectively. GFP-HJURP^{CDpoint} (top) (containing P229, R230, D254, C256, N257, D262, L263, Y264, M267 mutated to Alanine) or GFP-HJURP^{CDdel} (lacking residues N231 through D253) is sufficient to induce precocious CENP-A assembly (bottom).

Our combined results indicate that the conserved domain of HJURP is a cell cycle control element that interacts with cyclin A and that the RxL motif

critically contributes to this interaction. Importantly, disruption of this site is sufficient to alleviate at least part of the Cdk-mediated inhibition upon an otherwise functional HJURP.

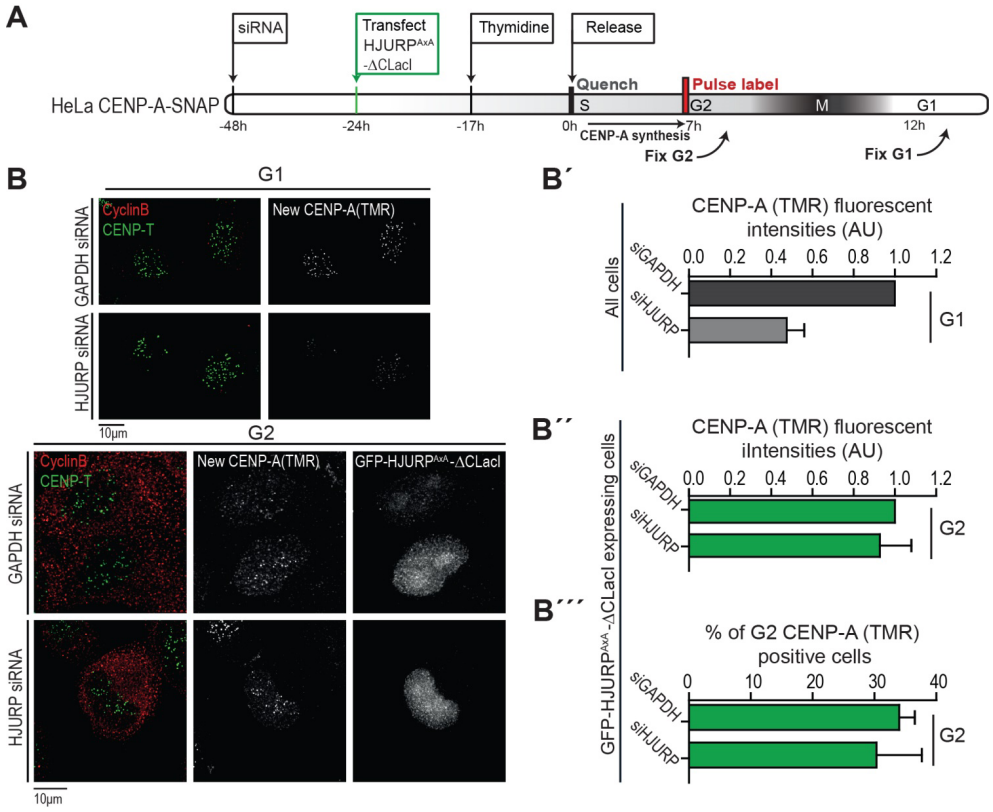


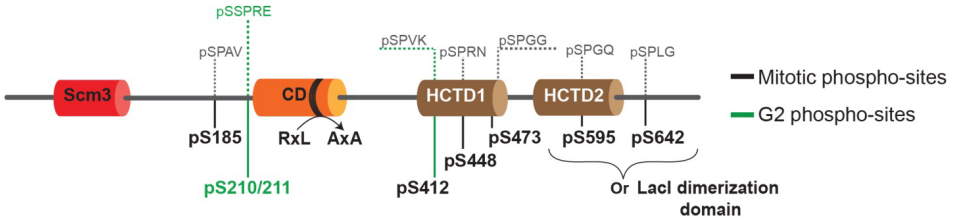
Figure 3.8 HJURP^{AxA} is a functional assembly factor. (A) HeLa CENP-A-SNAP cells were treated with siRNAs against HJURP or GAPDH and synchronized by double thymidine arrest and release combined with SNAP quench-chase pulse labeling as indicated. Cells were transfected with GFP-HJURP^{AxA}-ΔCLacI 24 hours prior to synchronous release into S phase. Cells were either fixed at G2 or cycled into the next cell cycle and collected at early G1 phase, following canonical CENP-A assembly. (B) Representative images of experiment described in (A). Cells were counterstained with Cyclin B, CENP-T and DAPI to indicate cell cycle status, centromeres and DNA respectively. (B´) CENP-A SNAP (TMR) fluorescent signal intensities of G1 cells are plotted in grey scale. Signals are normalized to siGAPDH. (B´´) CENP-A SNAP (TMR) fluorescent signal intensities of GFP-HJURP^{AxA}-ΔCLacI

Figure 3.8 Continued: expressing G2 cells are plotted in green. Signals are normalized to siGAPDH. (B´´´) Percentage of total G2 cell population positive for CENP-A-SNAP for indicated siRNA conditions were determined from 3 replicate experiments, plotted in green. All error bars indicate SEM.

HJURP Serine 210/211 is functionally phosphorylated in G2 phase cells

Next, we determined whether the uncoupling of HJURP from its cell cycle control involves specific phosphorylation sites. Previous study (Müller et al., 2014) identified S412, S448 and S472 located within HCTD1 domain of HJURP as key phosho-residues controlling HJURP centromere targeting in U2OS CENP-A-SNAP cell line. These residues along with three additional ones were identified in our SILAC approach. Expression of HJURP in which the 6 identified mitotic putative Cdk phospho-residues (Figure 3.2C) were mutated to alanine (either all 6 or combinations thereof) did not result in changes in the timing of CENP-A assembly in our HeLa-CENP-A-SNAP cell line (Figure 3.9A, B). One key difference between our study and the one performed by (Müller et al., 2014) is the type of human cells used along with their respective levels of CENP-A-SNAP transgene. Whereas U2OS cell used in (Müller et al., 2014) contain high levels of CENP-A-SNAP, HeLa cells used in our study display a much lower levels (~4-fold) (Figure 3.9C). Therefore, it is likely that this discrepancy is due to the strong overexpression of CENP-A in the Müller et al line. Consistently, when expressing HJURP^{S412A,S448A,S412A} in the same cell line as used in the Müller study we can recapitulate low levels of assembly (Figure 3.9D, D´).

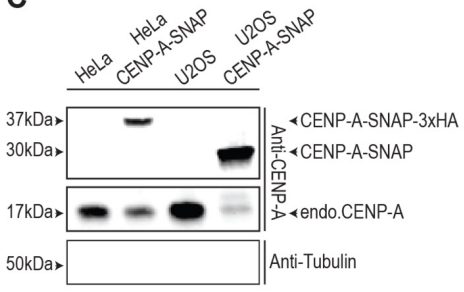
A



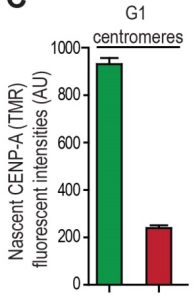
B

HJURP	G2 phase CENP-A assembly % intensity of G1 (% of G2 cells assembling)	
Mitotic phospho-site mutations	**C-terminal domain**	
	endogenous	**ΔC-LacI**
S185A	undetectable	undetectable
S185A, S412A, S448A,S473A	undetectable	undetectable
S595,S642A	undetectable	n.a.
S185A,S595A,S642A	undetectable	n.a.
S412,S448A,S473A	undetectable	undetectable
S185A,S412A,S448A,S473A,S595A,S642A	undetectable	n.a.
G2 phospho-site mutations		
S210A/S211A/S412A	~10% (~7%)	~10% (~15%)
S210A/S211A	undetectable	undetectable
Conserved domain mutation		
RxL>AxA	~30% (~25%)	~40% (~30%)
Other		
RxL>AxA, S412A (Figure 8C)	~30 (~50%)	n.d.

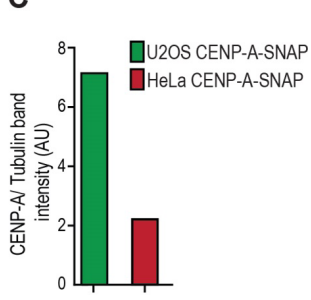
C



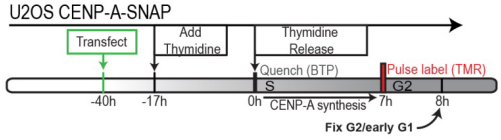
C´



C´´



D



D´

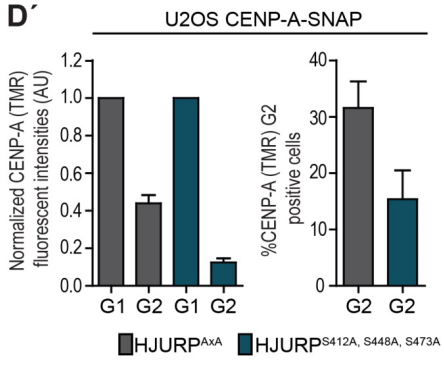


Figure 3.9 Summary of premature CENP-A assembly phenotypes of HJURP phospho-site mutants. (A) Schematic representation of HJURP protein, along with previously Figure recognized domains (CENP-A binding domain (Scm3), Conserved Domain (CD), HJURP C-Terminal Domain 1 and 2 (HCTD1 and 2)) and the position of phospho-sites identified by mass spectrometry in mitosis (black lines; Figure 3.2) or in G2 enriched cells (green lines; Figure 3.11). Amino acid sequences flanking phospho-sites are annotated. Position of LacI dimerization domain replacing the endogenous C-terminal dimerization domain is indicated. (B) Table summarizing premature CENP-A assembly phenotypes upon expression of indicated mutant HJURP proteins. Experiments were performed as in Figure 3.6. HeLa CENP-A-SNAP cells were transiently transfected with either GFP-HJURP or GFP-HJURP-ΔC-LacI and congenic point mutations thereof. 23h-post transfection, cell were enriched in G2 phase by a single Thymidine block, followed by 7h of release and subsequent fixation. CENP-A assembly was assayed using SNAP TMR-labeling of its S phase synthesized pool. Following fixation, cells were counterstained for cyclin B and DAPI to indicate G2 status and DNA, respectively. Efficiency of CENP-A assembly is indicated as % of G1 phase CENP-A intensities and % of transfected cells loading. undetectable: no centromere signals were discernable. n.d. not determined. n.a. not applicable. (C) Western blot showing higher levels of CENP-A-SNAP transgene in U2OS cell line (Müller et al., 2014) compared to HeLa cell line (used in this study). Extracts of randomly cycling U2OS CENP-A-SNAP and HeLa CENP-A-SNAP cell lines together with respective parental cell lines (carrying no transgene) were separated by SDS-PAGE followed by immunoblotting with anti-CENP-A and anti-Tubulin antibodies. (C´) Quantification of G1 CENP-A-SNAP fluorescent intensities from HeLa CENP-A-SNAP and U2OS CENP-A-SNAP. Randomly cycling HeLa CENP-A-SNAP and U2OS CENP-A-SNAP were subjected to Quench-Chase-Pulse experiment (as in Figure 3.6). CENP-A-SNAP (TMR) fluorescent signal intensities were determined using CRaQ method. (C´´) Quantification of CENP-A-SNAP band intensities of Western blot showed in (C) using Odyssey infrared scanner. Band intensities of CENP-A-SNAP were normalized to tubulin (loading control). (D) Low level of premature CENP-A assembly is induced by expression of GFP-HJURP^{S412A,S448A,S473A} mutant in U2OS CENP-A-SNAP cell line (Müller et al., 2014). U2OS CENP-A-SNAP cells were transiently transfected with indicated constructs. CENP-A assembly was assayed as described in Figure 3.6. (D´) Left: Quantification of CENP-A-SNAP (TMR) fluorescent signal intensities of cells from experiment described above in G2 phase (Cyclin B positive) and G1 phase (Cyclin B negative) as described in Figure 3.6. 3 replicates, error bars indicate SEM. Right: Quantification of frequency of pre-

Figure 3.9 Continued: -mature CENP-A loading in Cyclin B positive cells expressing either GFP-HJURP^{AxA} (grey) or GFP-HJURP^{S412A,S448A,S473A} (blue) from 3 replicate experiments.

However, expression of the HJURP^{AxA} mutant containing an Alanine substitution of Serine 412, resulted in an increased frequency and efficiency of precocious CENP-A deposition compared to expression of HJURP^{AxA} alone (Figure 3.10) in our HeLa-CENP-A-SNAP cell line. This further prompted us to test the casual link between decreased Cyclin A binding and HJURP phosphorylation status. Given that the cell cycle uncoupled-HJURP^{AxA} mutant has lost its interaction with cyclin A, but not cyclin B, we aimed to identify further potentially relevant phospho-residues in G2 phase, the cell cycle window in which we observe pre-mature CENP-A assembly. To this end, we expressed Doxycycline inducible 3xFlag-HJURP-ΔCLacI or 3xFlag-HJURP^{AxA}-ΔCLacI for 24h, specifically in G2 pha-

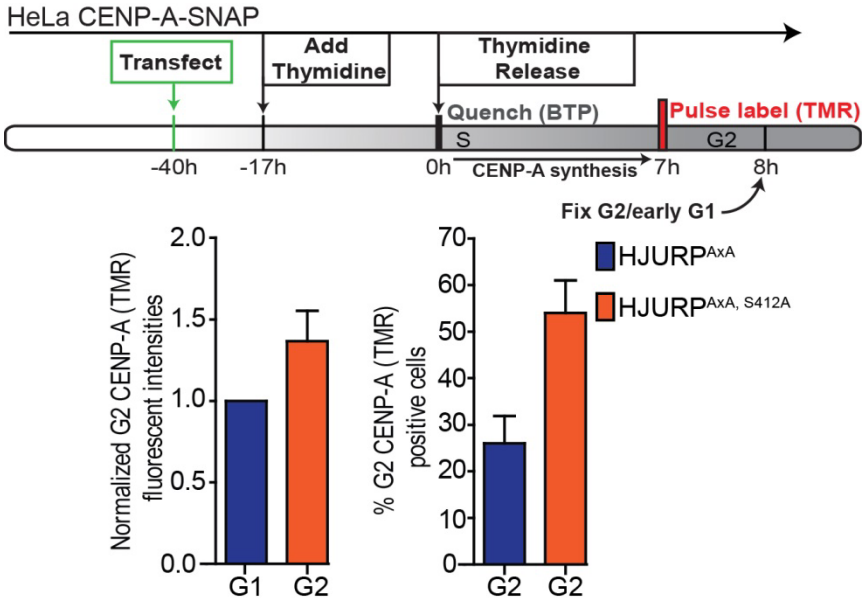


Figure 3.10 Expression of HJURP^{AxA,S412A} enhances premature CENP-A assembly. HeLa CENP-A-SNAP cells were transiently transfected with indicated constructs and

Figure 3.10 Continued: Thymidine synchronized to enrich cells in G2 phase, while CENP-A assembly was assayed using SNAP TMR-labeling of its S phase synthesized pool. Lleft: Quantification of CENP-A-SNAP (TMR) fluorescent signal intensities of cells from experiment described above in G2 phase (Cyclin B positive) and G1 phase (Cyclin B negative), using CENP-T signal as a centromere reference (not depicted) and CraQ. 3 replicates, error bars indicate SEM. Right: Quantification of frequency of premature CENP-A loading in Cyclin B positive cells expressing either GFP-HJURP^{AxA} (blue) or GFP-HJURP^{AxA,S412A} (orange). 3 replicates, error bars indicate SEM.

-se enriched HeLa HILO cells (Khandelia et al., 2011) (Figure 3.11A). Following immunoprecipitation, purified 3xFlag-HJURP was subjected to TiO2 phospho-enrichment and mass spectrometry to identify phosphorylated residues (Figure 3.11A, B). We identified S210/S211 phosphopeptides (the proximity of these residues prevented us from differentiating S210 vs. S211 as the site of phosphorylation (Figure 3.11D). Interestingly, these phosphopeptides were not detected in mitotically synchronized cells (Figure 3.2C). This suggests differential phosphorylation of HJURP, consistent with our finding that cyclin B also interacts with HJURP but in a conserved domain-independent manner (Figure 3.4C). Furthermore, the only common phospho-residue between G2 and mitotically synchronized cells was on serine 412 (Figure 3.11F). Importantly, when comparing the relative abundance of S210/S211 phospho-peptides we find these to be substantially reduced on the HJURP^{AxA} mutant in which cyclin A binding is reduced compared to wildtype. This suggests that the cyclin A/Cdk complex interaction with HJURP results in phosphorylation of this site (Figure 3.11D, E).

To test the functional significance of these residues we mutated serines 210 and 211 in combination with serine 412 and expressed HJURP^{S210A,S211A,S412A}-ΔCLacI mutants in G2 phase cells (Figure 3.12). Quench-chase-pulse labeling of CENP-A-SNAP in these cells showed that

mutation of the G2 phase residues to alanine results in low, but detectable levels of nascent CENP-A at centromeres (Figure 3.12B´). This indicates

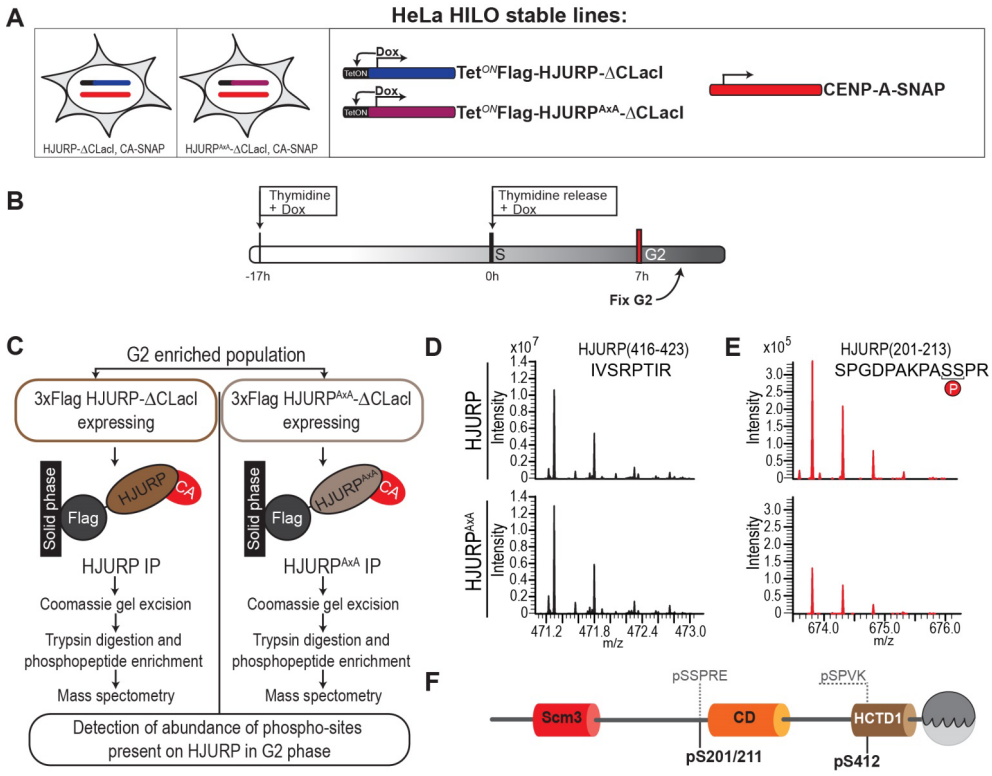


Figure 3.11 HJURP Serine 210/211 is phosphorylated in G2 phase cells. (A) Schematic of cell lines used for a label free mass spec analysis. (B) HeLa HILO cells carrying indicated Doxycycline-inducible HJURP constructs were enriched in G2 cells by Thymidine arrest and release during Dox induction. (C) Cell pellets obtained from experiment in (B) were subjected to immunoprecipitation using Flag-coupled agarose beads to isolate 3xFlag-HJURP-ΔCLacI, separated on SDS-PAGE followed by Coomassie-based excision of HJURP proteins. Purified proteins were subjected to trypsin digestion, phosho-peptide enrichment, followed by LC-MS/MS analysis. (D) Mass spectra of a representative non-phosphorylated HJURP peptide from the flow through of the phospho-enrichment, of samples from cells containing WT HJURP-ΔCLacI (top) and HJURP^{AxA} -ΔCLacI (bottom). (E) Mass spectra of the phosphopeptide containing pS210/pS211, from the elution of the phospho-enrichment from cells expressing indicated constructs. Because the two serines

Figure 3.11 Continued: are adjacent, it was not possible to differentiate between S210 and S211 as the site of phosphorylation. (F) Schematic representation of Cdk-consensus phospho-sites detected on HJURP in G2 phase.

that inhibition of assembly is at least partially compromised. These results strongly suggest that cyclin A binding to HJURP in G2 phase results in inhibitory phosphorylation, in part on serines S210/211 and S412, preventing premature CENP-A assembly.

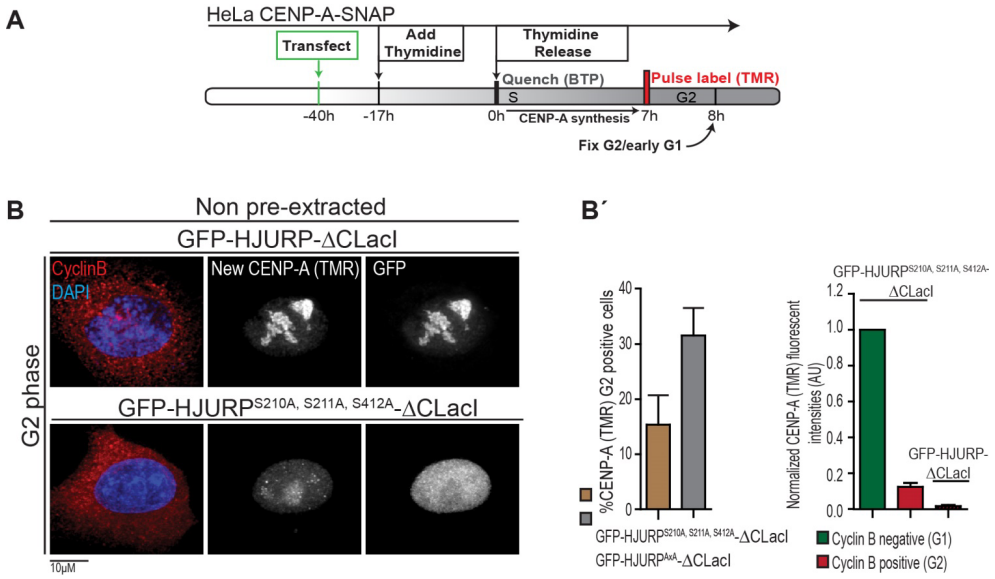


Figure 3.12. Serine 210/211 and S412 are critical residues within HJURP responsible for inhibition of CENP-A loading in pre-mitotic stages of the cell cycle. (A) Experiment analogous to Figure 3.6A, assaying indicated HJURP constructs for localization and CENP-A assembly in G2 phase. (B) Representative images of cells from experiment in (G). CENP-A assembly was assayed using SNAP TMR-labeling of its S phase synthesized pool. Following fixation, cells were counterstained for cyclin B and DAPI to indicate G2 status and DNA, respectively. (B´) Left: Quantification of frequency of premature CENP-A loading in Cyclin B positive cells expressing indicated constructs from 3 replicate experiments. Right: Quantification of CENP-A-SNAP (TMR) fluorescent signal intensities.

Cdk activity controls HJURP localization not its chaperoning activity

Phosphorylation of HJURP could directly interfere with its chaperoning activity, thereby inactivating the key function of the protein. Alternatively, it may sequester an otherwise active HJURP away from the centromere, preventing its untimely recruitment. To distinguish between these possibilities, we fused HJURP to the DNA binding domain of CENP-B (CBdbd) (Figure 3.13A). This domain binds specifically to centromeric α-satellite DNA and allows us to drive HJURP to centromeres in G2 synchronized cells, while likely bearing inhibitory phosphorylation due to high Cdk activity (Figure 3.13A). We detected nascent CENP-A-SNAP at G2 centromeres after expression of CBdbd-HJURP-GFP in approximately half the cells (Figure 3.13B) but not CBdbd-GFP alone, indicating centromeric localization of HJURP is sufficient to enable unscheduled CENP-A loading. Although HJURP is removed from mitotic chromatin (a process that apparently overrides the DNA binding activity of the CENP-B DNA binding domain), newly loaded CENP-A-SNAP remained associated with centromeres upon entry into mitosis, suggesting it is assembled into centromeric nucleosomes rather than part of an HJURP-associated prenucleosomal complex (Figure 3.13 right). Based on these results, we conclude that Cdk-driven phosphorylation does not interfere with HJURP chaperoning activity, rather it results in sequestering HJURP away from the centromere, preventing its untimely recruitment.

3.8 Discussion

In this chapter, I demonstrate that centromeric recruitment of CENP-A specific chaperone HJURP is negatively controlled by Cdk1/2 activities. Whereas HJURP is phosphorylated on consensus Cdk residues in G2 and M phase, upon mitotic exit, it is being rapidly dephosphorylated and

targeted to centromeres. We have mapped the putative Cdk phosphorylation sites on mitotic HJURP, some of which have previously been identified (Müller et al., 2014; Wang et al., 2014). In addition, we assign a novel function to the vertebrate conserved domain of HJURP as a

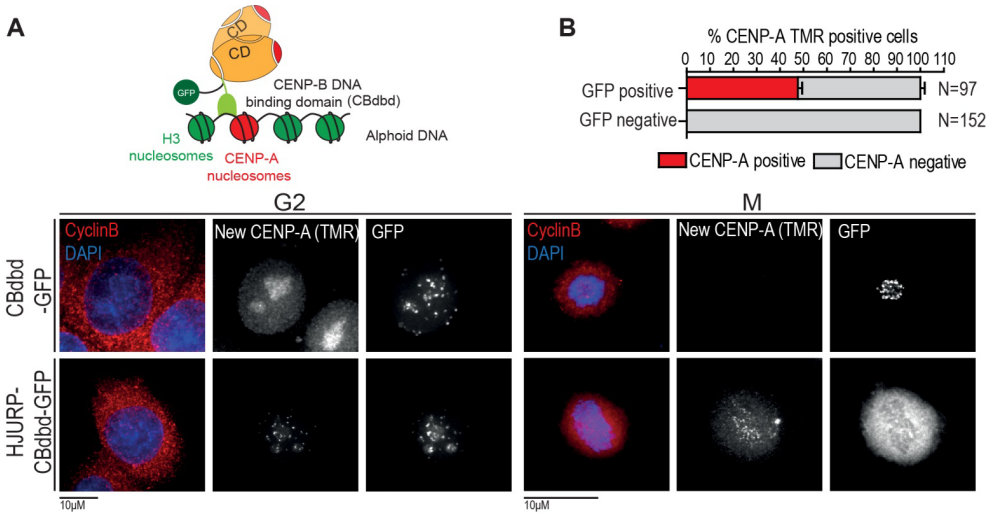


Figure 3.13 Cdk activity controls HJURP localization not its chaperoning activity. (A) Top: Schematic of relevant domains in centromere targeted HJURP. Bottom: HeLa CENP-A-SNAP cells were transfected with indicated constructs. 7 hours post Thymidine release cells were either fixed in G2 phase or collected in Nocodazole to enrich for mitotic cells. Cells were counterstained for cyclin B and DAPI to indicate G2 status and DNA, respectively. (B) Quantification of frequency of premature CENP-A-SNAP deposition in Cyclin B positive cells, driven by expression of Cbdb-HJURP-GFP. Error bars indicate SEM.

docking site for cyclin A/Cdk (Figure 3.4A, B). Importantly, we further show that the HJURP-Cyclin A interaction leads to phosphorylation of specific residues in G2 phase (Figure 3.11) that are required to maintain HJURP inhibition prior to mitosis (Figure 3.12). It is important to note here that even though we could detect low level of premature CENP-A assembly driven by HJURP^{S210A,S211A,S412A}-ΔCLacI in G2 cells, this does not necessarily mean that these three residues are the sole sites responsible for cell cycle-

dependent timing of CENP-A assembly. This is further exemplified by the higher frequency of premature CENP-A deposition caused by expression of HJURP$^{AxA,\ S412A}$ mutant. It is likely that there are other sites within HJURP which are contributing to its Cdk-dependent centromere localization and that their synergistic action is required for maintenance of this negative control. Nevertheless, Cdk 1/2-based phosphorylation of S210/S211 and S412 appear to be critical for complete sequestration of HJURP away from the centromere before mitosis. In mitosis, HJURP interacts with Cyclin B (in a conserved-domain independent manner) and is phosphorylated on yet different Cdk responsive residues compared to the ones detected in G2 phase. This suggests differential phosphorylation of HJURP as cells progress from G2 phase into mitosis. Mutations encompassing all mitotic phospho-residues were not sufficient, not only to induce premature loading of CENP-A in G2 phase, but also in mitosis. In addition, the HJURP^{6Ala} mutant displayed the same localization pattern in mitosis (excluded from chromatin) as a wild-type protein. It is possible that some of the sites that we have detected to be phosphorylated on HJURP in mitosis are acting as positive regulators of CENP-A deposition, thus being required for HJURP targeting to chromatin and subsequently, centromeres, upon mitotic exit. The strength of HJURP mitotic inhibition is also illustrated by its eviction from chromatin even though it has been previously artificially placed at the centromeres using Cbdb-tether in G2 phase. The prematurely loaded pool of CENP-A that we detect at the centromere in this case is likely to have been loaded in the preceding G2 phase. The fact that this CENP-A remains stably bound on mitotic centromeres indicates that it is being assembled into stable nucleosomes. This further suggest that, at least in G2 phase, HJURP retains the capacity to bind and deposit nascent CENP-A, but it is prevented to reach the centromere by direct Cdk1/2-dependent

phosphorylation. Of particular interest are also phospho-residues located within the C-terminal portion of HJURP, in the HCDT2 domain, which mediates HJURP dimerization. These two residues (S595 and S642) are most rapidly dephosphorylated after inactivation of Cdk1/2 activities, making them an attractive candidate controlling not only HJURP centromere targeting, but also HJURP dimerization, at least during mitosis. Since we have used a version of HJURP protein in which HCTD2 is replaced with the artificial LacI dimerization domain, we could not assess whether these residues are also phosphorylated in G2 phase. However, the fact that the HJURP^{AxA} mutant containing the wild-type dimerization domain is depositing CENP-A independently of endogenous HJURP, argues that in G2 phase this putative control of HJURP dimerization does not take place. The simplest explanation for this is that S595 and S642 are low-affinity sites for Cdk1/2, therefore only when Cdk1 reaches the peak of activity in mitosis, these residues are phosphorylated. Indeed, protein secondary structure and solvent accessibility prediction suggest that S595 is buried within a β-sheet whereas S210/211 are exposed at the surface of a β-strand (based on an algorithm provided by NetSurfP-Protein Surface Accessibility and Secondary Structure Predictions, Technical University of Denmark).

A role for NPM-1 in regulating CENP-A assembly?

Prior to its centromere targeting in G1 phase, HJURP is prominently enriched in G2 nucleoli (Dunleavy et al., 2009). However, one interesting and common feature of HJURP mutants that are driving premature loading of CENP-A is their dislocation from nucleoli in G2 phase despite high Cdk activities. Even though we could detect premature centromere targeting of HJURP^{AxA}, this was possible only after removal of soluble HJURP via pre-

extraction and amplification of the GFP-HJURP^{AxA} signal using GFP-booster. Still, even after this sample processing, the most prevalent localization of HJURP^{AxA} in addition to the centromere, was a large nucleoplasmic pool excluded from nucleoli. This suggests that sequestration of HJURP to nucleoli might be one of the steps ensuring its timely recruitment to centromeres. The strongest candidate-factor responsible for the putative sequestration of HJURP to nucleoli is NPM-1 (nucleophosmin-1). NPM-1 has numerous functions, some of which include histone-chaperoning activity, ribosome biogenesis and transport, centrosome duplication, etc (Lindström, 2011), and is localized prominently to nucleoli. In addition, NPM-1 is part of the CENP-A prenucleosomal complex, and has been detected in both CENP-A and HJURP purifications (Dunleavy et al., 2009; Foltz et al., 2009; Shuaib et al., 2010). In flies, nucleolar factor Modulo which is structurally related to nucleolin (a binding partner of NPM-1 in human cells), is reported to interact with CAL-1 (Chen et al., 2012). Depletion of Modulo results in CAL-1 delocalization from nucleoli, followed by a defective centromeric recruitment of CAL-1. Furthermore, Modulo together with NLP (nucleoplasmin-like protein) tether centromeres to the periphery of Drosophila nucleoli which contributes to the stable organization of pericentric chromatin (Padeken et al., 2013). Therefore, nucleoli in Drosophila have a dual (at least) role in maintenance of an active centromere: they direct the localization of CAL-1 and enable clustering of centromere at its periphery.

Based on these data, it is possible to envision a mechanism operating in human cells whereby NPM-1 sequesters nascent HJURP to nucleoli, adding another layer of control over temporally restricted CENP-A deposition. It would be interesting to test if inhibitory Cdk-dependent phosphorylation would stimulate the interaction between HJURP an NPM-

1, and alternatively, whether NPM-1-based nucleolar localization of HJURP is a prerequisite for inhibitory/activatory phosphorylation. However, given that our HJURP^{AxA} is active in CENP-A assembly even though it has (presumably) never been associated with nucleoli, it is likely that HJURP phosphorylation occurs prior to its association with nucleoli. Moreover, specific combination of inhibitory and activatory phosphorylation may be required for HJURP targeting to nucleoli. Therefore, a synergistic effect of both Cdk-driven phosphorylation and nucleolar sequestration would localize HJURP away from the centromeres, at least until mitotic entry. In mitosis, nucleoli are disassembled; hence the inhibitory mechanism exerted over HJURP would have to rely only on a highly active branch of Cdk inhibition, which is consistent with our data in which we could not, by any means, target HJURP to mitotic centromeres.

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CHAPTER 4

Full reconstitution of CENP-A assembly pathway under high Cdk1/2 activities

This chapter contains sections of the publication: Ana Stankovic, Lucie Y. Guo, João F. Mata, Dani L. Bodor, Xing-Jun Cao, Aaron O. Bailey, Jeffrey Shabanowitz, Donald F. Hunt, Benjamin A. Garcia, Ben E.Black, Lars E.T. Jansen. A Dual Inhibitory Mechanism Sufficient to Maintain Cell-Cycle-Restricted CENP-A Assembly, *Molecular Cell*, 65.2 (2017), 231–46

Abstract

Chromatin featuring the H3 variant CENP-A at the centromere is critical for its mitotic function and epigenetic maintenance. Assembly of centromeric chromatin is restricted to G1 phase through inhibitory action of Cdk1/2 kinases in other phases of the cell cycle. In this chapter, I describe our identification of the two key targets sufficient to maintain cell cycle control of CENP-A assembly. We uncovered a single phosphorylation site in the licensing factor M18BP1 (chapter 2) and a cyclin A binding site in the CENP-A chaperone, HJURP (chapter 3). Simultaneous expression of mutant proteins lacking these residues, results in complete uncoupling from the cell cycle. Consequently, CENP-A assembly is fully recapitulated under high Cdk activities, indistinguishable from G1 assembly. Finally, we show that displacement of M18BP1 from the centromere is critical for the assembly mechanism of CENP-A.

Introduction

Centromeres are chromosomal loci that drive faithful genome segregation during mitotic division (Allshire and Karpen, 2008). The functional foundation of the centromere is established by a specialized chromatin structure that features the histone H3 variant CENP-A (Black and Cleveland, 2011). CENP-A nucleosomes are at the core of a positive epigenetic feedback loop that maintains centromere identity. Consistent with this role, CENP-A nucleosomes are long lived and are maintained through multiple cell divisions (Bodor et al., 2013; Jansen et al., 2007). Its unusually slow turnover at each centromere (Falk et al., 2015) indicates that replenishment is either equally slow or is limited in time and tied to CENP-A redistribution following DNA replication. Indeed, in vertebrates, assembly of newly synthetized CENP-A is directly linked to cell cycle

progression and is initiated during mitotic exit and restricted to early G1 phase of the cell cycle (Jansen et al., 2007; Schuh et al., 2007).

Previously we showed that brief inhibition of cyclin dependent kinase 1 and 2 (Cdk1/2) activities is sufficient to drive CENP-A deposition prior to mitotic exit (Silva et al., 2012). This has led to a model where the CENP-A assembly machinery is present and poised for activity but is kept inactive throughout S, G2 and M phase, until mitotic exit when activities of Cdk1/2 drop, concomitant with the onset of CENP-A deposition. However, how this inhibition is achieved has been unresolved. An orchestrated action of several factors is necessary for the process of CENP-A deposition (Fukagawa and Earnshaw, 2014). Key proteins include the Mis18 complex and the CENP-A chaperone HJURP which bears CENP-A-specific nucleosome assembly activity (Barnhart et al., 2011; Dunleavy et al., 2009; Foltz et al., 2009; Fujita et al., 2007). HJURP and M18BP1 (also known as HsKNL2), a member of the Mis18 complex, are phosphoproteins (Bailey et al., 2015; Dephoure et al., 2008; Kato et al., 2007; McKinley and Cheeseman, 2014b; Müller et al., 2014; Silva et al., 2012; Wang et al., 2014) and localize to centromeres in a cell cycle controlled manner, in early G1 phase (Dunleavy et al., 2009; Foltz et al., 2009; Fujita et al., 2007; Maddox et al., 2007) indicating they are putative targets for Cdk regulation. In addition, recent work has identified the mitotic kinase Plk1 as a critical component to drive CENP-A assembly(McKinley and Cheeseman, 2014b). While Plk1 is itself a cell cycle controlled kinase, it does not restrict CENP-A assembly to G1 phase as it is required for both canonical assembly in G1 phase as well as for premature assembly upon Cdk inhibition. In addition, several residues on CENP-A itself are phosphorylated (Bailey et al., 2013, 2015; Yu et al., 2015; Zeitlin et al., 2001). One of these, serine 68, is proposed to be a Cdk target but its phosphorylation is restricted to mitosis,

and mutation of this residue does not lead to a change in the timing of CENP-A deposition (Yu et al., 2015). Furthermore, the role of this modification has been disputed (Fachinetti et al., 2017). In contrast, it has been reported that mutations of phospho-residues in HJURP or artificial recruitment of M18α to centromeres results in premature centromere recruitment of CENP-A (McKinley and Cheeseman, 2014b; Müller et al., 2014). While these studies point to a contributing role for these factors, the efficiency at which CENP-A assembly occurred is unexplored. This leaves open the critical question of which factors are necessary, which are sufficient, how Cdk-mediated control is exerted, and how key proteins are functionally inhibited. To resolve these specific molecular steps that ensure cell cycle restricted CENP-A assembly, we report full uncoupling of CENP-A assembly from the cell cycle/Cdk regulation. To achieve this, we identified a cyclin-interacting domain in HJURP (chapter 3 of this thesis) and a critical phospho-site in M18BP1 (chapter 2 of this thesis). Simultaneous uncoupling of these factors from cell cycle progression results in a complete reconstitution of CENP-A assembly process in G2 phase, prior to mitotic exit. Finally, we find that CENP-A assembly results in the active removal of the licensing factor M18BP1 that is functionally required for the completion of CENP-A assembly. Our results define a dual inhibitory mechanism that is sufficient to maintain cell cycle restricted centromere propagation and define the molecular underpinnings of how assembly is turned on and subsequently turned off.

Materials and Methods

DNA constructs

All DNA constructs used in this chapter were described previously in chapter 2 and chapter 3 of this thesis.

Cell lines and culturing conditions

All human cell lines used were grown at 37°C, 5% CO2. Cells were grown in DMEM (Bio West) supplemented with 10% fetal bovine serum (FBS) (BioWest)), 2 mM glutamine, 1 mM sodium pyruvate (SP) (Thermo Fischer Scientific), 100 U/ml penicillin, and 100 µg/ml streptomycin, with the exception of HeLa HILO derived cell lines in which 10% tet-free (BioWest) FBS was used. HeLa HILO RMCE cell lines were a gift from E.V. Makeyev, Nanyang Technological University, Singapore, and contain a single genomic recombination site which allows for the insertion of a tetracycline responsive expression cassette (Khandelia et al., 2011). The four lines outlined in Figure 4.2 were assembled as follows: HeLa HILO RMCE clone #10 (Khandelia et al., 2011), was transfected with (pLJ649) that constitutively drives GFP-M18BP1^{T653A} expression. Positive clones were selected with 500 µg/ml of Neomycin (Gibco). A polyclonal population was sorted based on GFP fluorescence. A single clone of HeLa HILO GFP-M18BP1^{T653A} as well as the parental HILO RMCE clone #10 were transduced with pBABE-CENP-A-SNAP-3xHA retrovirus (pLJ718) (Bodor et al., 2012). Infected cells were selected by 300 µg/ml of Hygromycine (Invitrogen). Individual resistant cells were sorted by FACS. CENP-A-SNAP-3xHA clones #9 and #10, respectively were selected for further analysis. This selection was based on equal expression of CENP-A-SNAP-3xHA between different cell lines, as determined by immunoblot using

rabbit anti-CENP-A (Cell Signaling technology) and by TMR fluorescent intensities. Both clones were then transfected with 2,5 ng/µl of pLJ745 and pLJ746, vectors carrying two loxP sites flanking the Doxycycline (Dox) inducible 3xFlag-HJURP or 3xFlag-HJURP^{AxA} expression construct. Cre recombinase (Khandelia et al., 2011) was added at 1% of total DNA content. Positive clones were selected using 1 µg/ml of Puromycin (MERCK). Expression of 3xFlagHJURP/HJURP$^{WT/AxA}$ was induced by 10 µg/ml of Doxycycline (Sigma-Aldrich) and assayed for equal expression by western blot using FlagM2 antibody (Sigma-Aldrich). U2OS CENPA-SNAP cell lines were gift from Genevieve Almouzni (Institut Curie, France).

Cell synchronization

Double Thymidine-based synchronization was performed as described (Bodor et al., 2012). For Mitotic synchronization, 2,4 µM of EG5 inhibitor III Dimethylenastron-DMEIII (Calbiochem) was used for 24h. For synchronous mitotic exit, following DMEIII washout, HeLa and Hek293T were released for 5h and 7h, respectively. For inhibition of proteolysis, 10µm of MG132 was used.

DNA transfection and siRNA treatment

Transient transfection of HeLa CENP-A-SNAP and HEK293T was performed using Lipofectamine LTX (Invitrogen; Carlsbad, CA) according to the manufacturer's instructions. All siRNAs were obtained from Dharmacon. Mis18α was depleted as previously reported (Silva et al., 2012).

SNAP Quench-Chase-Pulse Labeling

Cell lines expressing CENP-A-SNAP were pulse labeled as previously described (Bodor et al., 2012a), with exception of HeLa HILO derived cell

lines where BTP (New England Biolabs) concentration was adjusted to 0,5 µM.

Immunofluorescence and pre-extraction procedure

Procedures are essentially as described (Bodor et al., 2012a).

Microscopy

Imaging was performed using a DeltaVision Core system (Applied Precision) inverted microscope (Olympus, IX-71), coupled to a Cascade2 EMCCD camera (Photometrics). Images (ranfing from 512x512 to 1024x1024) were acquired at 1 x binning using a 100x oil objective (NA 1.40, UPlanSApo) with 0, 2 µm z sections.

DAPI area as a measure of cell cycle position

To identify G2 cells in experiment presented in Figure 6 we synchronised cells in early S phase (by double Thymidine block), G2 phase (by double Thymidine block and 7h of release), late G2 (by an overnight treatment with RO3306 (Roche)) or left them asynchronous. Using these synchronized populations we established a cut-off for DAPI area size of G2 cells for each experiment. Following image acquisition, thresholding parameters selecting isolated DAPI areas were manually adjusted using ImageJ software. Subsequently, these parameters were propagated to all data sets, and an average DAPI area of each cell cycle stage was determined. Cells were identified as G2 if DAPI area was at least two standard deviations above the average DAPI area size of the S phase population. We confirmed that these values completely overlapped to the averages of DAPI area size coming from G2 synchronized populations (double thymidine released or

RO3306 treated). All cells that had an equal or smaller DAPI area size from average values of S phase population were excluded from the analysis.

Results

Cdk-mediated control of M18BP1 and HJURP is sufficient to ensure tight cell cycle timing of centromere propagation

In the chapter 3 of this thesis, I described the identification of a conserved domain mutant of HJURP-HJURP^{AxA}, whose expression is sufficient to drive precocious assembly of CENP-A in G2 phase, indicating alleviation of Cdk1/2-based inhibition over this chaperone. Although HJURP^{AxA} is capable of inducing unscheduled CENP-A assembly, it does so with a relatively low efficiency and centromere specificity as compared to canonical G1 loading (Figure 3.6A). This indicates that an additional level of cell cycle control exists. A candidate for this is the Mis18 complex, which includes Mis18α, Mis18β and the associated protein M18BP1 (Fujita et al., 2007). All subunits share a common localization pattern, with highly enriched and centromere specific localization in anaphase, followed by disappearance in mid-G1 (Fujita et al., 2007; Silva and Jansen, 2009). Interestingly, we found that premature HJURP^{AxA} driven CENP-A assembly in G2 phase correlates with low levels of stably expressed GFP-Mis18α at centromeres (Figure 4.1A-C). Moreover, siRNA-mediated depletion of Mis18α leads to a loss of both canonical assembly in G1 phase as well as premature assembly of CENP-A in G2 phase (Figure 4.1D). This demonstrates that HJURP^{AxA}-induced assembly occurs through the canonical assembly pathway and suggests that the partial nature of this assembly is possible due to low levels of Mis18 complex members at G2 centromeres.

Previously, we have identified a single conserved phospho-residue (T653) within the largest member of the Mis18 complex, M18BP1 which is responsible for its cell-cycle dependent centromere recruitment (chapter 2).

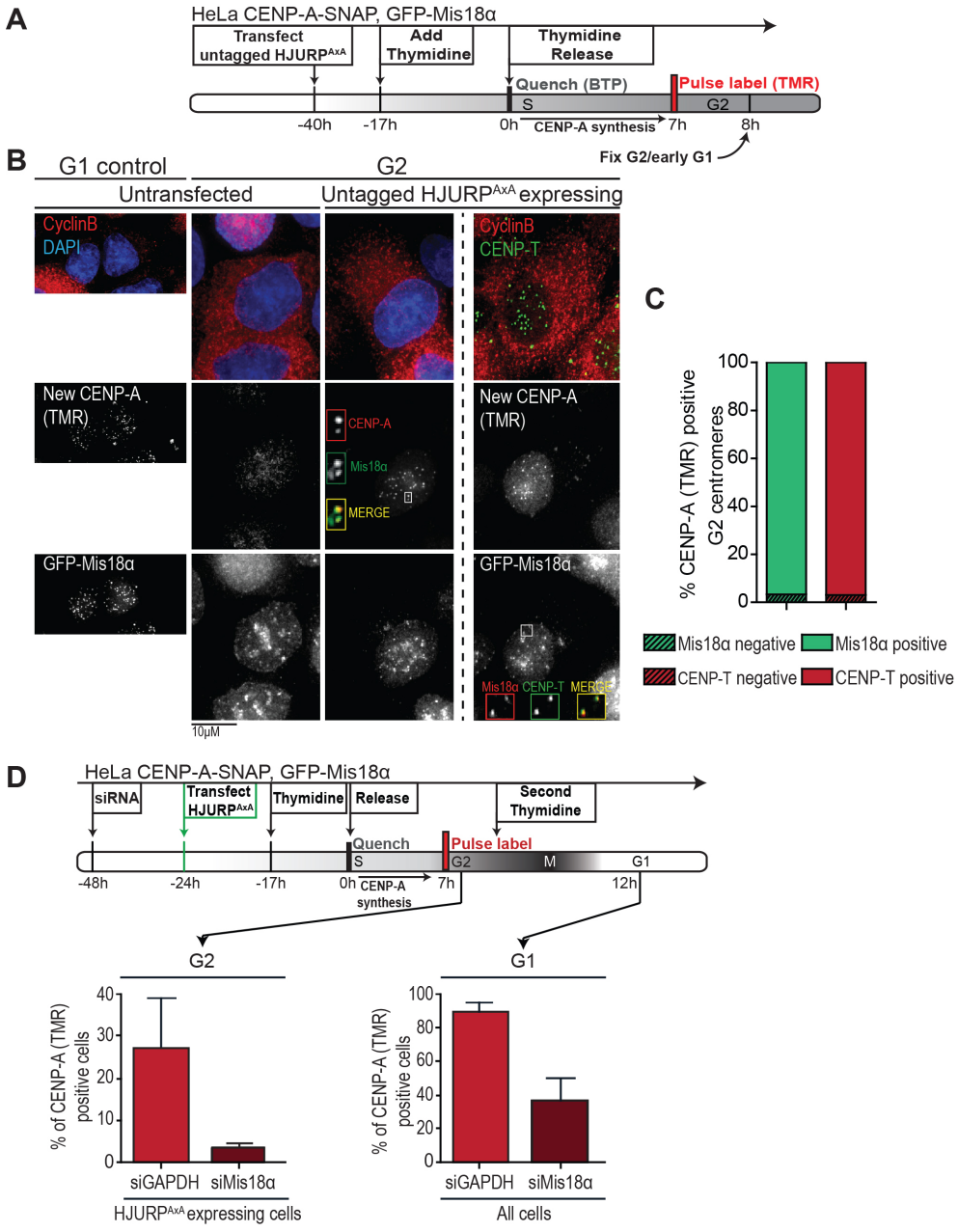

Figure 4.1 HJURP^{AxA} induced CENP-A assembly is Mis18α dependent. HJURP^{AxA} CENP-A assembly is Mis18α dependent. (A) Stable GFP-Mis18α, CENP-A-SNAP double transgenic HeLa cells were transfected with untagged HJURP^{AxA}, synchronized and

Figure 4.1 Continued: assayed for nascent CENP-A assembly by SNAP quench-chase-pulse labeling, followed by immunostaining for cyclin B and DAPI to indicate G2 status and DNA, respectively. (B) Representative images of experiment described in (A). (C) Quantification of frequency of CENP-A (TMR) positive G2 centromeres of experiment described in (A). Cells were scored in relation to whether GFP-M18α (green) or CENP-T (red) signals are simultaneously detected together with CENP-A (TMR) or not. (D) Top: Scheme outlining RNAi against Mis18α or GAPDH, synchronization and Quench-Chase-Pulse labeling of CENP-A-SNAP, GFP-Mis18α cells. (Bottom) Quantification of CENP-A-SNAP (TMR) positive cells from 3 independent experiments. Error bars indicate SEM.

Substitution of this residue with the one of Alanine is sufficient to prematurely target M18BP1 to G2 centromeres, and, provided constitutive expression, M18BP1^{T653A} can induce an infrequent and low-levels of unscheduled CENP-A assembly (chapter 2, Figure 2.5). Therefore, the results presented in chapters 2 and 3 of this thesis indicate that centromere localization of both HJURP and M18BP1 is blocked by Cdk-mediated phosphorylation, suggesting that combined phospho-control of these protein complexes contributes to cell cycle specific loading of CENP-A. To directly test this, we constructed a specific set of cell lines in which we can conditionally control CENP-A assembly with high temporal resolution (Figure. 4.2A). We used HeLa HILO cells in which we inserted an expression cassette at a defined genomic location by recombination-mediated cassette exchange (RMCE) (Khandelia et al., 2011). Using this system we expressed either HJURP-ΔCLacI or HJURP^{AxA}-ΔCLacI under the control of a doxycycline-inducible promoter. HJURP induction was performed either in cells stably expressing GFP-tagged M18BP1^{T653A} or expressing endogenous M18BP1. CENP-A assembly was determined by quench-chase-pulse labeling of stably expressed CENP-A-SNAP (Bodor et al., 2012). As observed after transient expression, induction of HJURP^{AxA} alone resulted in low levels of CENP-A assembly (Figure 4.2B, C).

Conversely, constitutive M18BP1^{T653A} expression only led to infrequent and inefficient recruitment of nascent CENP-A to G2 centromeres. Remarkably, induction of HJURP^{AxA} combined with stably expressed M18BP1^{T653A} resulted in highly efficient and centromere restricted CENP-A assembly in G2 phase. Careful quantification of fluorescent intensities of this G2 loaded CENP-A pool demonstrated that centromeric CENP-A levels led to 93% of G1 controls (Figure 4.2C). In sum, disrupting the timing of centromere targeting of either HJURP or M18BP1 results in a limited deregulation of CENP-A assembly, as has been shown previously (McKinley and Cheeseman, 2014; Müller et al., 2014), we were able to show that simultaneous uncoupling of both of these proteins leads to full-fledged CENP-A assembly, indistinguishable of the canonical assembly, strongly suggesting that M18BP1 and HJURP are the principal (if not the sole) targets of Cdk-mediated inhibition.

Efficient CENP-A assembly requires displacement of M18BP1 from the centromere

Mis18 proteins localize to centromeres in a unique temporal pattern, targeting in anaphase prior to the onset of CENP-A assembly and disappear in mid G1. This has led to the proposal that the Mis18 complex is part of a priming step in CENP-A assembly (Fujita et al., 2007; Fukagawa and Earnshaw, 2014). Interestingly, we observed that induction of CENP-A assembly in G2 phase resulted in concomitant loss of centromeric GFP-M18BP1^{T653A} levels to under 30% relative to the uninduced control (Figure 4.2D). Expression of HJURP^{AxA}, but not wild-type HJURP results in GFP-M18BP1^{T653A} loss, showing that displacement is directly dependent on CENP-A assembly. This suggests that M18BP1 removal is an active, CENP-A loading-dependent process and not a passive consequence of cell

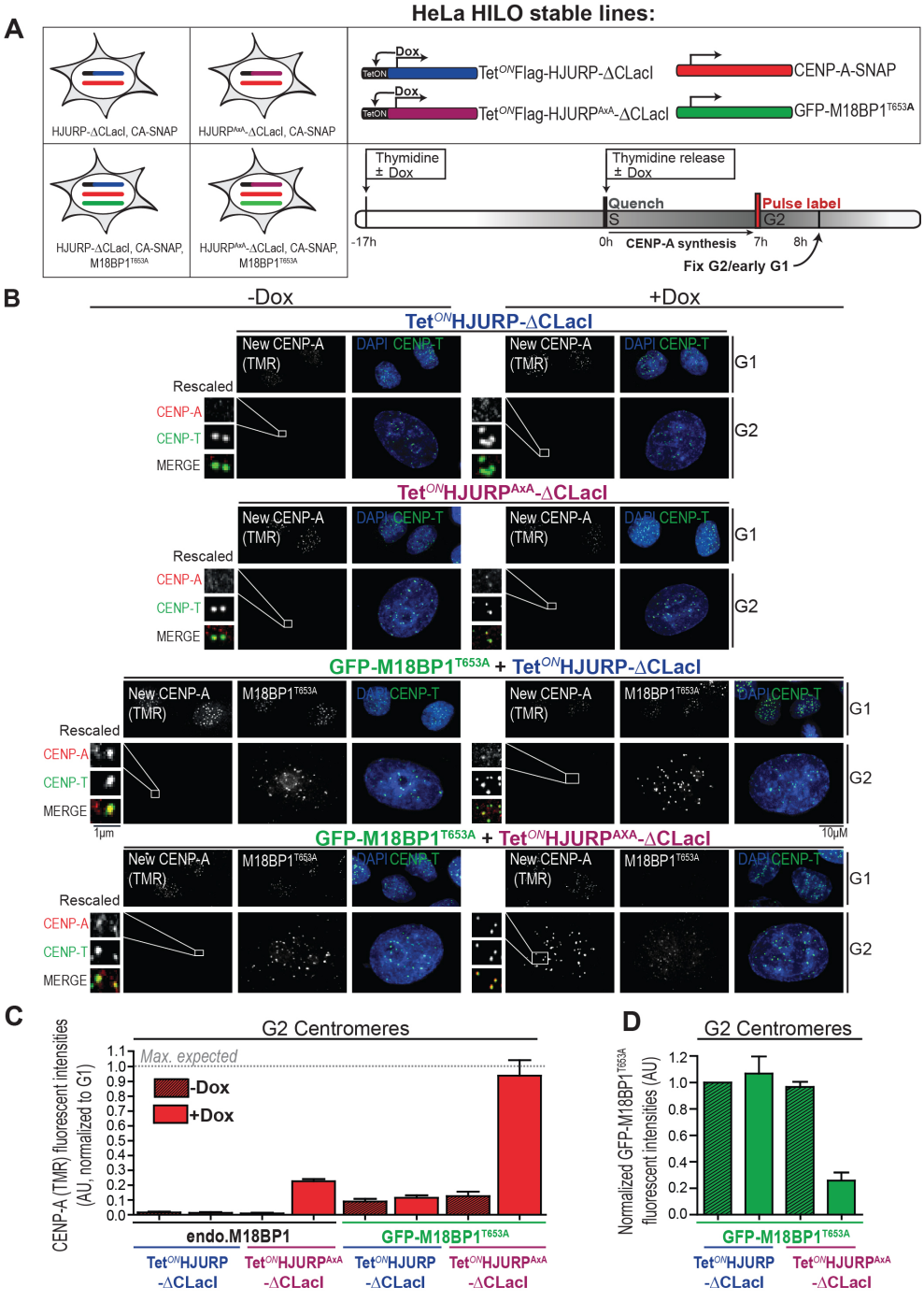
A
HeLa HILO stable lines:
HJURP-ΔCLacI, CA-SNAP
HJURPAxA-ΔCLacI, CA-SNAP
HJURP-ΔCLacI, CA-SNAP, M18BP1T653A
HJURPAxA-ΔCLacI, CA-SNAP, M18BP1T653A
Dox
TetON
TetONFlag-HJURP-ΔCLacI
CENP-A-SNAP
TetONFlag-HJURPAxA-ΔCLacI
GFP-M18BP1T653A
Thymidine ± Dox
Thymidine release ± Dox
Quench
Pulse label
S
G2
-17h
0h
CENP-A synthesis
7h
8h
Fix G2/early G1
B
-Dox
+Dox
TetONHJURP-ΔCLacI
TetONHJURPAxA-ΔCLacI
GFP-M18BP1T653A + TetONHJURP-ΔCLacI
GFP-M18BP1T653A + TetONHJURPAXA-ΔCLacI
New CENP-A (TMR)
DAPI CENP-T
M18BP1T653A
Rescaled
CENP-A
CENP-T
MERGE
G1
G2
1μm
10μM
C
G2 Centromeres
CENP-A (TMR) fluorescent intensities (AU, normalized to G1)
Max. expected
-Dox
+Dox
endo.M18BP1
GFP-M18BP1T653A
TetONHJURP -ΔCLacI
TetONHJURPAxA -ΔCLacI
D
G2 Centromeres
Normalized GFP-M18BP1T653A fluorescent intensities (AU)

Figure 4.2 A dual inhibitory mechanism restricts CENP-A deposition to G1 phase. (A) Schematic representation of Hela HILO cells constitutively expressing low levels of CENP-A-SNAP (red), with or without stable expression of GFP-M18BP1T653A (green) along with Tetracycline inducible 3xFLAG-HJURP-ΔCLacI (blue) or 3xFLAG-HJURP^{AxA}-ΔCLacI (purple). Cell lines were synchronized and Dox induced as indicated followed by labeling of an S phase synthesized pool of CENP-A-SNAP of by a single Thymidine block, and released into G2 phase where an was labelled. (B) Images of CENP-A-SNAP assembly. After fixation, cells were counterstaining for CENP-T and DAPI to indicate centromeres and DNA, respectively. Cell cycle status was determined by measuring total DAPI area (see methods). Insets of individual centromeres are rescaled to visualize weak signals. (C) Quantification of CENP-A-SNAP fluorescent signals from B. Average CENP-A-SNAP signals from G2 centromeres were normalized to G1 centromeres, and corrected for centromere number (assuming signal intensity per focus represents 1 and 2 centromeres in G1 and G2, respectively). Error bars indicate SEM of 4 independent experiments. (D) Quantification of centromeric GFP-M18BP1^{T653A} fluorescent signals from B. Intensities were compared between lines constitutively expressing GFP-M18BP1^{T653A} and inducible 3xFLAG-HJURP-ΔCLacI or 3xFLAG-HJURP^{AxA}-ΔCLacI. Average GFP-M18BP1^{T653A} signals were normalized to uninduced 3xFlag-HJURP-ΔCLacI expressing cells. Error bars indicate SEM of 4 independent experiments.

cycle progression.

Further, treatment with the general proteasome inhibitor MG132 results in high levels of M18BP1^{T653A} at G2 centromeres (Figures 4.3A, B), indicating that M18BP1^{T653A} displacement is driven by proteasome-dependent consumption. Strikingly, stabilization of M18BP1^{T653A} results in a reduction of CENP-A assembly (Figures 4.3A, B), suggesting that removal of M18BP1 is required for completion of CENP-A assembly. However, this result can be interpreted in several ways. In addition to preventing the loss of M18BP1, MG132 broadly blocks proteolysis, preventing us from establishing a causatively link between the maintenance of M18BP1 at centromere with the inability to load CENP-A. Therefore, we cannot exclude

that the inhibition of proteolysis leads to an impairment of CENP-A assembly independent of higher M18BP1 levels at the centromere.

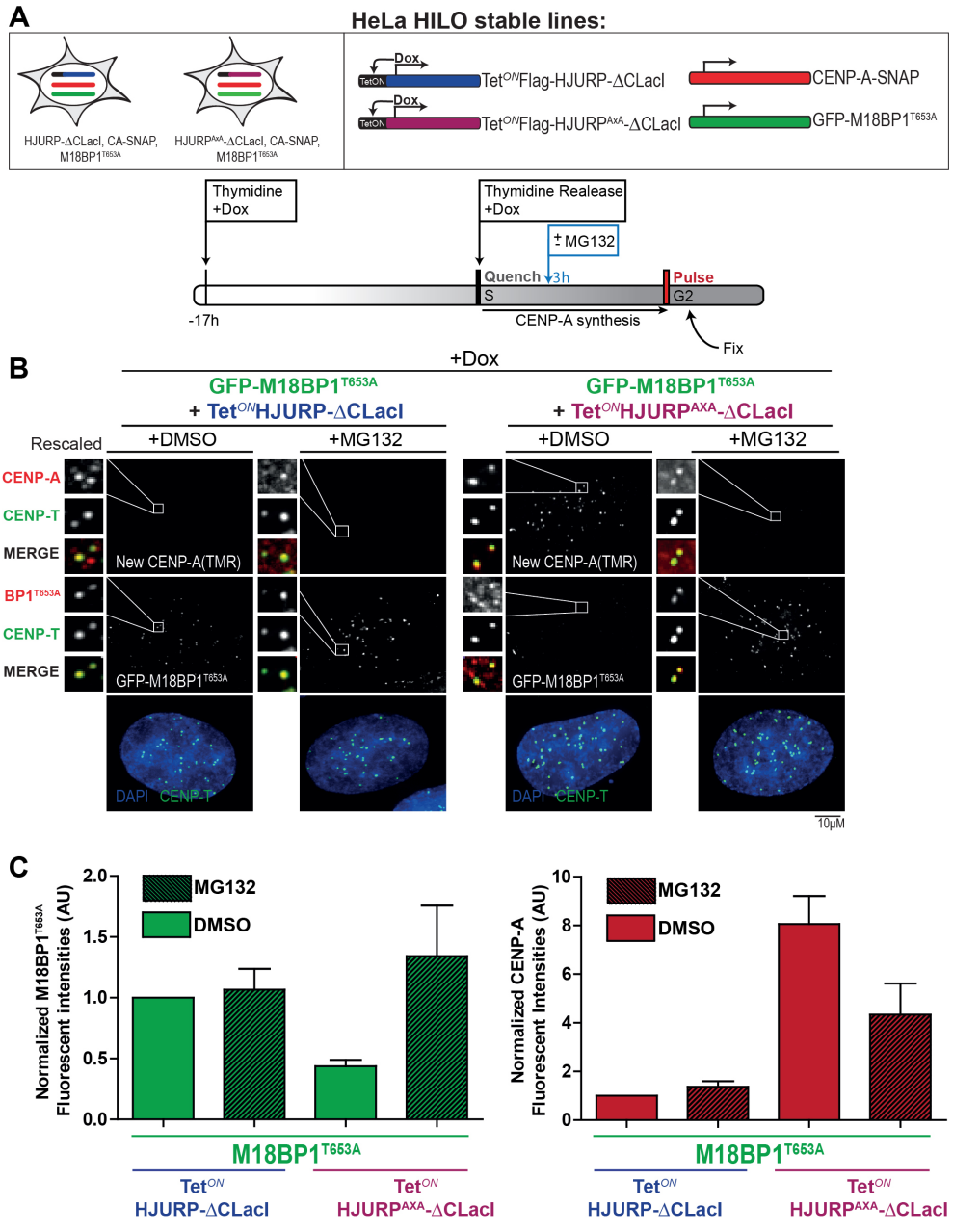

Figure 4.3 M18BP1 turnover at centromeres is necessary for efficient unscheduled CENP-A assembly. (A) HeLa HILO cell lines constitutively expressing GFP-M18BP1^{T653A} together with inducible 3xFLAG-HJURP-ΔCLacI or 3xFLAG-HJURP^{AxA}-ΔCLacI, were synchronized with a single Thymidine block, released into G2 phase, when a nascent, S/G2 phase synthesized pool of CENP-A-SNAP was labelled. Doxycycline was added together with Thymidine to all cell lines, and maintained throughout the experiment (24h). Following 4h after release from single Thymidine either 10µm MG132 or DMSO was added and maintained until the end of experiment. (C) Quantification of experiment outlined in B (images shown in B). Centromeric G2 signals of GFP-M18BP1^{T653A} (green) and CENP-A-SNAP (red) for each cell line are plotted. Average signal intensity of each measurement was normalized to its respective DMSO treated 3xFLAG-HJURP-ΔCLacI cells.

To directly test the contribution of M18BP1 centromere residence time in G1 cells, we either over-expressed wild type M18BP1 or artificially tethered it to the G1 centromeres (using the CBdbd tether) while assaying for CENP-A assembly (Figure 4.4). We observe a ~40% reduction in nascent CENP-A fluorescent intensities in either of these conditions. We conclude that while M18BP1 is an essential positive regulator of CENP-A assembly, preventing its turnover by overexpression or by rendering it unable to be removed from G1 centromeres results in defects in CENP-A assembly.

Discussion

We have identified the two key targets of Cdk-based inhibition during pre-mitotic phases of the cell cycle that are sufficient for maintenance of strict cell cycle control of CENP-A assembly; the licensing factor M18BP1 and the CENP-A chaperone HJURP (Figure 4.5). We defined residues in both factors that are responsible for their cell cycle control, mutation of which renders the entire assembly machinery essentially insensitive to the inhibitory pre-mitotic state (Figure 4.2). In addition, we provide a causal link between efficient CENP-A assembly and M18BP1 displacement from the

centromere (Figure 4.3 and 4.4), indicating that M18BP1 is functionally consumed during the CENP-A loading process. Importantly, Both M18BP1

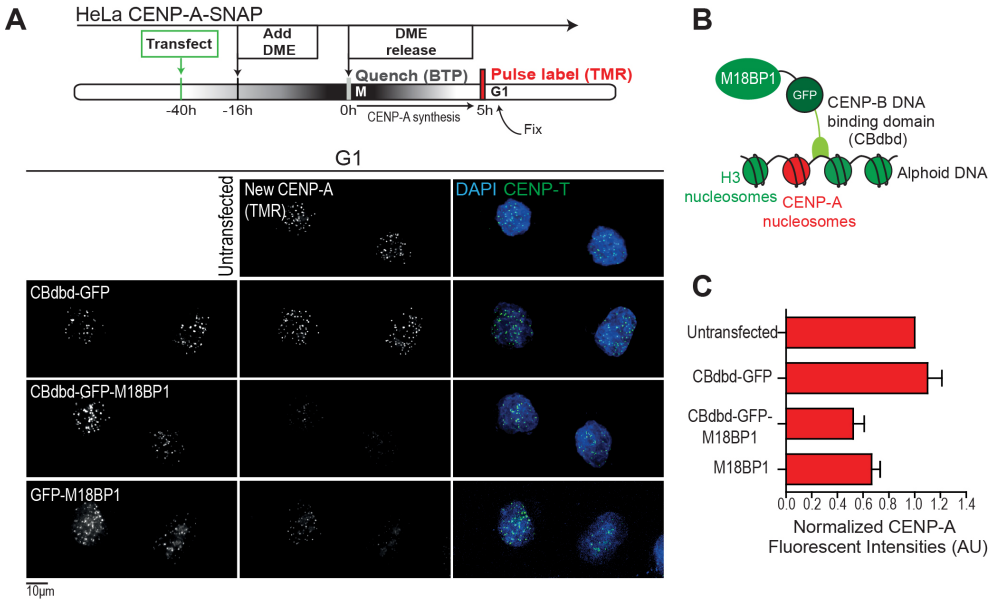

Figure 4.4 M18BP1 turnover at centromeres is necessary for efficient CENP-A assembly in G1 phase. (A) HeLa CENP-A-SNAP cells were transfected with indicated constructs, and synchronized in mitosis by an overnight treatment with Eg5 inhibitor (DME). A G1 synthesized CENP-A pool was labelled in mid G1 with TMR. GFP positive cells were analysed for CENP-A TMR fluorescent intensities. (B) schematic of CBdbd-GFP-M18BP1 fusion protein bound to alphoid DNA. (C) quantification of experiment outlined in A.

and HJURP are functional under high Cdk activity and have the capacity to form relevant complexes and to load CENP-A when artificially placed at the centromere (Figures 2.5 and 3.12). This indicates that the primary mechanism of Cdk-mediated inhibition is to prevent otherwise active factors from reaching the centromere (Figure 4.4). We propose that phosphorylation blocks the ability of M18BP1 and HJURP to bind to a partner(s) already docked at the centromere. M18BP1 interacts with CENP-C which is a constitutive core component of the centromere (Dambacher et

al., 2012; Hori et al., 2013; Moree et al., 2011; Nardi et al., 2016; Stellfox et al., 2016).

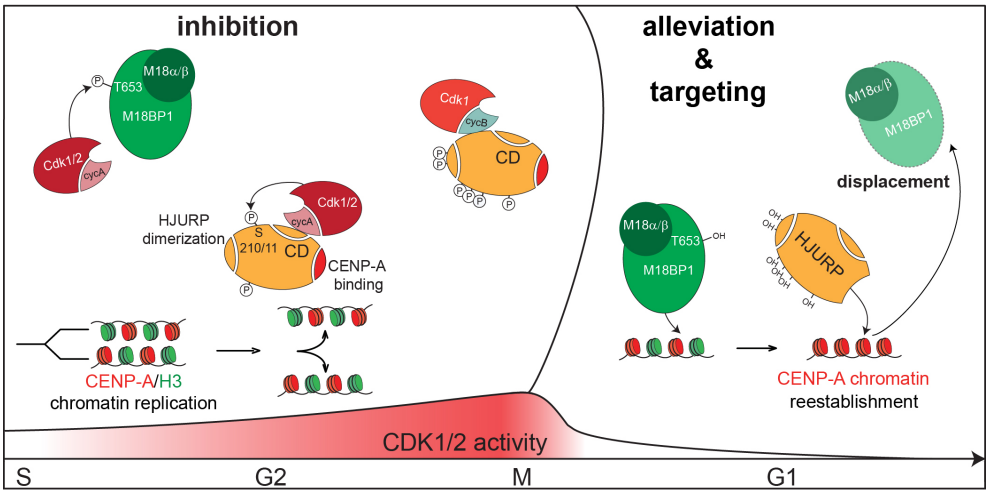

Figure 4.5 Model summarizing the key molecular steps that are sufficient to restrict CENP-A assembly to G1 phase. Inhibition: In S/G2 phase M18BP1 is phosphorylated at Cdk sites that include T653 which prevents it and its interaction partners Mis18α and β from localizing to the centromere. Similarly, centromere targeting of HJURP is blocked through phosphorylation via an interaction of CyclinA/Cdk with the HJURP conserved domain (CD). Alleviation and targeting: Upon loss of Cdk activity during mitotic exit, both proteins are dephosphorylated and targeted to centromeres, initiating CENP-A assembly. Following priming, active removal of M18BP1 is required for the completion of the assembly process.

In turn, the prenucleosomal HJURP/CENP-A complex binds to the Mis18 complex (Nardi et al., 2016; Wang et al., 2014). Our proposal is consistent with a recent report describing an interaction between HJURP and the Mis18 complex subunit Mis18β that is reduced upon Cdk phosphorylation, in vitro (Wang et al., 2014). While several recent studies have reported cell cycle regulated phosphorylation of CENP-A itself (Yu et al., 2015) or Plk1-mediated modification of M18BP1 (McKinley and Cheeseman, 2014), neither of these impact on the timing of CENP-A assembly. Therefore,

while key positive regulatory events may involve phospho-regulation, we identified the specific targets and mechanisms of the inhibitory control that is responsible for restricting CENP-A assembly to G1 phase. Inhibition of CENP-A assembly prior to mitosis at the level of HJURP or M18BP1 alone is incomplete. An HJURP mutant with a reduced cyclin A interaction leads to partial CENP-A assembly, while abrogation of M18BP1 phospho-control leads to low levels of CENP-A incorporation. This is in agreement with previous studies that showed that mutation of HJURP phospho-sites within the centromere targeting domain (HCTD1) (Müller et al., 2014) or forced recruitment of M18BP1 resulted in precocious CENP-A assembly (McKinley and Cheeseman, 2014). In sum, we find that rather than relying on a single tightly regulated factor, the combinatorial action of two layers of control synergize to efficiently restrict CENP-A assembly to early G1 phase.

The designation of the Mis18 complex as a priming (licensing) factor was originally inspired by its temporal centromere localization that initiates in anaphase, before the onset of CENP-A assembly (Fujita et al., 2007). This is analogous to the timing of chromatin targeting of the pre-replication complex (pre-RC) in early G1 phase which licenses DNA replication in the subsequent S phase (Nishitani and Lygerou, 2002). We now suggest that the commonalities between the licensing process of DNA replication and centromeric chromatin replication are greater than previously anticipated. A single round of genome duplication per cell cycle is achieved by the removal of licensing proteins from chromatin during S phase, as a direct consequence of DNA replication (Blow and Dutta, 2005; Blow and Hodgson, 2002).

By analogy, we find that removal of M18BP1 from the centromere is directly coupled to the onset of CENP-A deposition, at least under induced

conditions in G2 phase. Artificial placement at the centromeres or over-expression of M18BP1 greatly reduces the efficiency of canonical CENP-A assembly (Figure 4.4). This provides a causal link between efficient CENP-A assembly and M18BP1 displacement from the centromere. These results reveal novel parallels between DNA replication and CENP-A-chromatin, manifested in consumption of the licensing factor which is directly instigated by the start of duplication of the heritable mark. These findings are consistent with a recent study showing that nascent CENP-A/HJURP binding to the Mis18 complex in vitro leads to disassembly of the Mis18 complex (Nardi et al., 2016), leading to the proposal that Mis18 complex disassembly could be a mechanism to turn off CENP-A chromatin assembly. Our data on the removal of Mis18BP following CENP-A assembly in vivo provides additional direct evidence for this model. We show that not only does CENP-A assembly result in Mis18 complex removal (as shown by (Nardi et al., 2016)) but that this is a requirement for complete loading of CENP-A. Two possible implications follow from these observations. First, while M18BP1 is required for recruitment of nascent CENP-A to centromeres, its presence may block completion of the assembly process. By direct binding to CENP-C (Dambacher et al., 2012; Moree et al., 2011; Shono et al., 2015; Westhorpe et al., 2015) which in turn interacts with CENP-A (Falk et al., 2015; Guse et al., 2011; Kato et al., 2013; Logsdon et al., 2015), it is possibly that M18BP1 physically marks the site of incorporation for nascent CENP-A. Inability to remove M18BP1 would therefore provoke steric inhibition, resulting in low rates of CENP-A incorporation. Secondly, given the key role in initiation of CENP-A loading, removal of M18BP1 from centromeres provides an ´´OFF´´ switch for the process of assembly, thereby contributing to a tight cell cycle window ensuring a single round of CENP-A incorporation per cell cycle.

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CHAPTER 5

General Discussion:

Histone-based Inheritance, Centromeres and Evolution

Abstract

In the first chapter of this thesis, I described our current understanding regarding the basic properties of the epigenetically encoded centromere. The basic heritable unit of the centromere is the histone variant CENP-A, that constitutes a heritable epigenetic mark. In this chapter, I attempt to extend concepts of epigenetic inheritance beyond the CENP-A based centromere, describing other examples of histone-based epigenetic inheritance, with the goal of deciphering intercepting pathways that may be common for the majority of histone-based epigenetic inheritance. In that regard, I focus in particular on the maintenance of CENP-A nucleosomes across the cell cycle, where I suggest that the same mechanisms allowing recycling of canonical parental histones at replication fork may be acting in the case of CENP-A as well. Additionally, I discuss the emergence of the epigenetic centromere from an evolutionary point of view, and provide a speculative idea on how new centromeres could be arising and evolving. Before delving into these subjects, I will be briefly discussing the basic concepts of epigenetic inheritance from a historical perspective, as it provides the foundation for our modern understating of epigenetic phenomena.

5.1 The phenomenon of epigenetic inheritance

The ´´modern synthesis´´ evokes a gene-centric view of evolution whereby random mutation would lead to gradual adaptation and evolutionary change driven by natural selection (Fisher and A., 1930; Huxley, 1944). However, genes, as defined by DNA sequences, account only for a small subset of the heritability of an associated phenotype, indicating the existence of an additional heritable factor(s), which contribute to overall phenotype and associated adaptive fitness of a given population (Jirtle and Skinner, 2007;

Manolio et al., 2009; Noble, 2013). An alternative mode of inheritance to traditional Mendelian one is impelled by non-genetic, including epigenetic means (Pigliucci et al., 2010) Epigenetic inheritance could be defined as information that cell can pass to their progeny without changing their DNA sequence (Jablonka and Lamb, 2005). A heritable epigenetic mark is a stable carrier of epigenetic information that contains an intrinsic capacity to self-propagate independent of continuous input of the originating signal, while being steadily maintained throughout organismal lifespan. A heritable epigenetic mark can also be seen as a sensor of the external environment or of developmental cues which confers memory of a previous state. Importantly, if all memories of one generation are propagated to the succeeding one, the epigenetic system becomes non-selective. Therefore, the ability to ´´forget´´ is as important as the one to ´´remember´´, which is exemplified by the global genome remodelling upon fertilization to generate a totipotent cell state and the remodelling of the primordial germ cells during early embryogenesis. Additionally, this mode of inheritance is context dependent, and in a true Lamarckian spirit, it has to confer a certain evolutionary advantage to the organism in order to survive natural selection and become a heritable trait (Burkhardt and Jr., 2013; Haig, 2007). An often misunderstood and over-simplified Lamarckian theory of evolution proposed that individual interaction with the environment is an important component of heredity and evolution. Through use and disuse, individuals gain or lose characteristics during their lifetime and characteristics acquired during an individual's lifetime are inherited from one generation to the next. These are the two tenets of what is today known as the theory of inheritance of acquired traits. The most controversial aspect of this theory is that it implies that information embedded in somatic cells can influence the one stored in germline. This was the main reason why August Weismann

staunchly dismissed Lamarck´s use/disuse theory, while advocating for one-directional flow of information from germ to somatic cells, in what was to be known as Weismann´s barrier (Knight et al., 1889; Parker et al., 1893). According to him, germ cells are resistant to environmental and somatic ques, and thus it would be impossible for acquired traits to be inherited by future generations. In support of his theory, he showed that artificial shortening of mouse tail was not inherited by its offspring. However, while this experiment simply proved that not “any” acquired trait will be inherited, it did not test for the broader biological significance of acquired traits which was an integral part of Lamarck´s theory. Interestingly, Darwin, in a general sense, supported Lamarck’s theory, and actually introduced a hypothesis called “pangenesis” in his book Variation in Plants and Animals under Domestication (Darwin and Gray, 1868). According to Darwin, environmental cues induce somatic cells to shed microscopic ´´gemmules´´ or ´´pangenes´´, which circulate and accumulate in germ cells. The gemmules, in essence, transfer information from somatic cells to germ cells, thus affecting the next generation. Today, we know that in effect such phenomena do exist. One clear example is the CRISPR (Clustered regularly interspaced short palindromic repeats)-Cas immune system in bacteria. CRISPR-Cas is the adaptive and heritable immune system of prokaryotes. Bacteria who encounter a phage infection during their lifespan, will, if surviving the attack, integrate the genomic signature of the virus into their own CRISPR genomic locus (equivalent to their heritable germ line), in order to fend off future attacks. Therefore, the CRISPR-Cas system represents a non-Mendelian form of acquired inheritance that is highly adaptive. This is consistent with the idea that epigenetic variation could provide a selective advantage in fluctuating environment while compensating for a low-rate of genetic changes (Richards, 2008). If a

population would solely rely on a constant rate of genetic changes, an adaptation would be hampered by reduced genetic variation associated with bottlenecks or founder effects. Epigenetic changes would allow short-term adaptation to the new environment, which may be selected for and ultimately be subjected to genetic fixation. This phenomenon has been observed in the house sparrow (*Passer domesticus*), which shows increased phenotypic and epigenetic diversity and decreased genetic diversity after its introduction to a new niche (Liebl et al., 2013).

5.2 Histone based epigenetic inheritance

As long as the epigenetic trait is self-sustaining in the absence of the originating stimulus, it can be categorized as epigenetically inherited. This can be attained through self-propagating transacting mechanisms (*trans*), or by *cis*-acting molecular signatures physically associated with the DNA sequence/chromatin region they regulate (Allis and Jenuwein, 2016). *Cis* epigenetic signals are physically associated and inherited along with the chromosome on which they act. This comprises covalent modification of the DNA itself, such as DNA methylation, along with histones that can carry information in their primary sequence (histone variants) or in post-translational modifications often present on their N-terminal tails. Here, I will be discussing the basic properties of histone-based epigenetic inheritance. Much of the debate regarding if histones and their modifications are true carriers of epigenetic information stems from long-standing question as to if and how histones (old versus new) are segregated at the replication fork. Initial studies using pulse-chase methods to track the dynamics of chromatin-bound H3 and H4 histones suggested that at the bulk level, parental histones do not mix with newly synthetized ones (Jackson and Chalkley, 1974), and are randomly distributed between the two daughter

chromosomes (Jackson and Chalkley, 1985). More recent studies using a sophisticated mass spec-based approach, confirmed that the canonical H3 is not inherited through a half-nucleosome template, indicating that there is no splitting of parental H3-H4 tetramer (Xu et al., 2010). Additionally, isolation of newly replicated chromatin revealed equal distribution of old H3, and its associated methyl marks to daughter DNA strands (Alabert et al., 2014). Newly synthesized H3 does not contain these methyl marks when deposited, resulting in a dilution of cellular levels of methylated H3 right after replication. Along this line, post-translational modification of histones (PTM) are maintained in front of replication fork and serve as a template for modification of newly deposited histones which ultimately share the same pattern of PTM as the parental ones (Alabert et al., 2015). This is in contrast to the levels of H2A.Z variant which levels drop much more compared to H3.3, indicating lack of inheritance of this histone variant in the daughter chromosomes (Alabert et al., 2014). These studies have given rise to the proposal of a histone based mode of inheritance, in which histones and their post-translational modifications, following initial establishment can perpetuate their own inheritance through complexes that recognize a specific histone variant or modification on an inherited parental histone (factors named ´´readers``) and catalyse the same type of modification on adjacent newly deposited nucleosomes (´´writers´´) (Allis and Jenuwein, 2016). This model implies that kinetics of histone turnover is slow enough to allow recruitment of downstream modifying enzymes that specifically recognize retained histones or their post-translational modification. Is this ´´histone-based´´ inheritance autonomous with regard to DNA sequence? For example, in *S. pombe*, formation of pericentromeric heterochromatin depends on small-interfering RNAs (siRNAs). These siRNAs are produced from noncoding centromeric RNAs (ncRNAs) that are

transcribed from the underlying DNA repeats and loaded onto the RNA-induced initiator of transcriptional silencing (RITS) complex (Verdel et al., 2004; Volpe et al., 2002). This complex in turn provides binding specificity for CLRC complex, which contains the Clr4/Suv39h histone H3 lysine 9 (H3K9) methyltransferase. Importantly, stable binding of the RITS complex to chromatin requires Clr4 which recruits chromodomain of Chp1 to bind to nucleosomes containing H3K9me (Bayne et al., 2010; Gerace et al., 2010). However, even though siRNAs provide necessary specificity for histone-modifying enzymes, their input is continuously required to maintain the silencing of chromatin domain. This is exemplified by an experiment in which an artificial hairpin complementary to ura4+ gene was initially sufficient to drive silencing of this locus, but the effect was lost after several generations upon removal of the hairpin (Iida et al., 2008; Simmer et al., 2010). Likewise, in *S. cerevisiae*, deletion of genetic elements called silencers, responsible for attracting histone deacetylase SIR complex, causes a rapid loss of silencing after one cell division (Cheng and Gartenberg, 2000; Holmes and Broach, 1996). In Drosophila, establishment and maintenance of embryonic gene expression pattern requires binding of Polycomb group of proteins to specific regulatory sequences, called Polycomb response elements (PREs) (Ringrose and Paro, 2004). Excision of PREs results in the loss of silencing (hypoacetylation/methylation of H3K27) of a reporter white^{+} gene (Busturia et al., 1997; Sengupta et al., 2004). These results suggest that specific genomic loci can drive their own epigenetic state which is achieved through recruitment of effector factors that confer differential pattern of histone modifications, resulting in the silent transcriptional state of a given locus. Importantly, maintenance of silent state is dependent on a continuous input from genetic regulatory elements, arguing against DNA-independent, histone-based inheritance. However,

inducible artificial targeting of HP1α (Heterochromatic protein 1) to the upstream region of euchromatic Oct4 locus in mouse embryonic stem cells (ES) is sufficient to induce formation of heterochromatic H3K9me3 domain and consequently silencing of Oct4 gene (Hathaway et al., 2012). Remarkably, conditional removal of HP1α had no effect on preservation and propagation of heterochromatic domain, which was heritably transmitted across multiple cell generations. This is indicative of the formation of a positive epigenetic feedback loop which is self-sustainable and does not require a constant input of the initiating signal. The observed propagation of silent states is reminiscent of a classical example of silencing of the CD4 gene during differentiation of T cell lineage. Helper cells $CD4^+$ and $CD8^+$ killer cells are part of the adaptive immunity in mammals, and originate from the same precursor cell which expresses both CD4 and CD8 at its surface (double positive-DP cells) (Ellmeier et al., 1999). Cell type-specific expression of the CD4 receptor is regulated by a transcription silencer called *Cd4* silencer located in the first intron of the *Cd4* gene. The Cd4 silencer is inactive in DP cells, which allows for CD4 expression. During development of CD8 lineage, the transcription repressor Runx3 is expressed, which binds to the Cd4 silencer driving Cd4 repression in the developing CD8 cells (Collins et al., 2009; Taniuchi and Littman, 2004). Importantly, once established, the repression is self-perpetuated independently of the Cd4 silencer, such that in mature CD8 cells, the Cd4 silencer can be conditionally deleted without de-repressing Cd4, even following numerous rounds of cell divisions (Taniuchi et al., 2002; Zou et al., 2001). Thus, similarly to HP1α induced silencing of Oct4 locus, the Cd4 silencer is required for the establishment, but not the maintenance or mitotic propagation of CD4 repression. Using an analogous principle to (Hathaway et al., 2012), two recent studies from Moazed (Ragunathan et

al., 2014) and Allshire (Audergon et al., 2015) laboratories highlighted self-reinforcing properties of histone based inheritance. Using *S. pombe* as a model system, authors firstly established ectopic H3K9me repressive marks at the reporter locus via tethering of unique budding yeast histone-methyl-transferase Clr4. Upon removal of tethered Clr4, repressive histone marks were rapidly lost across cell divisions, analogous to the hairpin induced silencing mentioned above. However inactivation of the putative histone demethylase Epe1 upon Clr4 release resulted in stable maintenance and transmission of repressive chromatin marks, not only through mitotic, but remarkably, through meiotic divisions as well. Importantly, the bromodomain of Cl4 responsible for recognition of H3K9me3 mark was crucial for maintenance of repressive chromatin state. These studies demonstrate that histones with associated repressive marks indeed bear capacity to drive their own inheritance through direct ´´read´´ and ´´write´´ mechanism, whereas a counterforce of ´´eraser´´ (histone demethylase in this case) restricts their inheritance. Remarkably, a study in *C. elegans* demonstrated that repressive H3K27me histone marks inherited from paternally supplied X chromosomes are transmitted through the germ line into the offspring where they persist through several cell divisions (Gaydos et al., 2014). Notably, these marks drive the perpetuation of a repressed X state by serving as a template for PRC2 (Polycomb repressive complex 2) mediated formation of repressed chromatin state. Similarly, paternally derived centromeric CENP-A nucleosomes serve as a template for establishment of centromeres on paternally-derived chromosomes in developing embryo (Raychaudhuri et al., 2012). Depletion of paternal CENP-A nucelosomes results in failure of centromere establishment on paternal genome, in spite of the presence of maternally supplied CENP-A molecules and centromeric DNA on paternal chromosomes. Therefore, the

most common mode of histone-based propagation of epigenetic traits consists of their establishment by a transient initiation signal, followed by their conversion into *cis*-acting epigenetic signatures that bear autonomous self-propagating properties.

5.3 Maintenance of CENP-A nucleosomes across the cell cycle

At the heart of the epigenetic inheritance of the centromere is a specialized histone variant, CENP-A. Incorporation of CENP-A into centromeric nucleosomes generates a stable epigenetic mark that provides a heritable signal for incorporation of nascent CENP-A molecules each time when a cell divides. How the process of CENP-A assembly is regulated, along with critical factors involved in this process is described in chapters 1, 2, 3 and 4 of this thesis. However, incorporation of nascent CENP-A requires a template, upon which new molecules will be assembled. This platform is constituted of parental CENP-A nucleosomes, that have been assembled in the previous cell cycle and maintained throughout cell division. How the process of maintenance of CENP-A nucleosomes actually occurs along with critical factors contributing to its stable chromatin residence have remained largely undefined. One of the biggest questions in this regard is how do CENP-A nucleosomes survive the passage of replication fork during DNA replication in S phase. As for canonical histones, their replenishment behind the replication fork is achieved through combination of *de novo* histone deposition and recycling of old histones (Probst et al., 2009). One critical difference between the dynamics of canonical nucleosomes and CENP-A containing one is the absence of *de novo* CENP-A deposition in S phase in majority of animal species. Consequently, CENP-A nucleosomes have to remain stably associated with centromeres through S, G2 and mitotic stages of the cell cycle, in order to mark an

active centromere in proceeding G1 phase. This implies that during the passage of replication fork, parental CENP-A molecules have to be recycled and re-deposited at centromere. Recently, it has been shown that recycling of parental H3-H4 histones depends on MCM2, a key subunit of the replicative helicase (Huang et al., 2015). The current model of histone recycling suggests that, as the replication fork progresses, parental nucleosomes are being disrupted, with H3-H4 tetramers evicted from DNA and captured by MCM2. Subsequently, the MCM2-$(H3\text{-}H4)_2$ complex is recognized by AsfI, the H3-H4 histone chaperone, which binding disrupts the H3-H4 tetramer (Alabert et al., 2015; Groth et al., 2007; Huang et al., 2015; Loyola et al., 2006). Subsequently, AsfI alone or in cooperation with another histone chaperone (such is CAF-I) could deposit parental H3-H4 histones on newly synthetized DNA. Interestingly, crystal structure data revealed that MCM2 can bind all H3 variants (H3.1, H3.2, H3.3) including CENP-A (Huang et al., 2015), raising the attractive possibility that the handling of histones by MCM2 represents a common first step in the recycling mechanism genome wide, including the centromere. It would be of great interest to determine if MCM2 is indeed involved in the recycling of parental CENP-A nucleosome, and if true, which histone chaperone is involved in their re-incorporation. The CENP-A specific chaperone HJURP may the most obvious candidate (Dunleavy et al., 2009; Foltz et al., 2009), however other histone chaperone, such is DAXX, has also been reported to bear the capacity to incorporate CENP-A containing nucleosome particle, albeit in conjunction with H3.3 (Lacoste et al., 2014). Irrespective of the mechanism involved in the maintenance of parental CENP-A nucleosome, this question is particularly important if considered in the context of maintenance of the correct CENP-A levels. Current evidence indicates that existing centromeric CENP-A is redistributed stochastically during DNA

replication. The ratio in pool size between two sister centromeres follows a normal distribution averaging at 50/50 with a certain probability that one daughter centromere inherits a disproportionally larger (or smaller) number of parental CENP-A nucleosomes (Bodor et al., 2014). Importantly, the amount of CENP-A inherited from the previous generation will directly impact the amount of the one deposited by the daughter centromere. This notion is supported by the fact that the amount of CENP-A present at the centromeres is in a direct proportion to varying total cellular levels (Bodor et al., 2014), suggesting that the CENP-A loading machinery is not a rate-limiting factor controlling the size of centromeric domain, rather, it is CENP-A itself. The challenge to our understanding of how CENP-A levels are maintained is the fact that the chromatin bound pool does not exchange, rendering it invisible to a classic equilibrium. There is no apparent communication between soluble and centromeric CENP-A. This indicates that cells need some other measure of how much CENP-A is in chromatin and to adjust the assembly accordingly, since too little CENP-A would render centromeres dysfunctional [e.g. reducing CENP-A levels to 10% is ultimately incompatible with viability of cells (Black et al., 2007)], while too much CENP-A can potentially lead to neocentromere formation as is the case in Drosophila (Heun et al., 2006; Olszak et al., 2011). Given the nature of a positive feedback loop, in the absence of a dynamic equilibrium, individual centromeres would have the potential of reaching extreme values, spinning out of control unless there is a mechanisms to curb the assembly of new CENP-A. In addition, due to the nature of chromatin recycling during DNA replication, including a stochastic redistribution of CENP-A during S phase, CENP-A levels would be increasingly variable. It is conceivable that there are surveillance mechanisms which would monitor and sense imbalanced number of CENP-A nucleosomes at each

centromere. One possibility is that the CENP-A assembly machinery would incorporate a pool of molecules not in a direct relation to the number present in chromatin but load in excess, which has been observed (Jansen et al., 2007; Lagana et al., 2010). In this scenario, the correct amount would be determined in a later ‘’maturation’’ step, in which the overloaded pool of new CENP-A would be removed from the centromere having an excess of parental CENP-A, whereas those with reduced levels would be stripped to a lesser extent (Figure 5.1). Should there be such an eviction mechanism, it would have to allow discrimination between CENP-A marked for instability versus the one which is destined to be stably inherited over cell cycle. Whereas molecular steps allowing eviction of the overloaded pool of CENP-A are largely unknown, there are reports of stabilization of nascent CENP-A occurring in G1 (Lagana et al., 2010; Liu and Mao, 2016; Perpelescu et al., 2009), suggesting that addition of CENP-A ´´stabilization´´ mark would happen prior to DNA synthesis. Centromeric CENP-A levels could also be normalized during S phase passage, in which the mix of parental and G1-loaded pools of CENP-A would be coordinately and preferentially segregated to the grand-daughter centromere which inherited a decreased number of CENP-A molecules from the previous generation. An elegant model has been proposed linking the amount of CENP-A assembly in G1 phase directly to the strength of the centromere in mitosis (Brown and Xu, 2009). In this model, weaker centromeres would bind a smaller number of microtubules that would in turn generate a signal driving the assembly of a compensatory number of CENP-A molecules in the subsequent G1 phase. One drawback of this model is that it assumes a proportional nature of kinetochore assembly in relation to the number of CENP-A molecules. However, variations of this model could be extended to modular kinetochores (assembled in a fixed rate independently of the

number of CENP-A nucleosomes). Assuming nearly-equal numbers of microtubules attached to each daughter centromere (due to checkpoint signaling), the signal required to stabilize the amount of CENP-A molecules would come from the tension generated within centromeric chromatin. A speculative idea is that only those CENP-A molecules that are under tension are marked for stability whereas superfluous ones are marked for removal. In this way, over multiple mitotic divisions the number of CENP-A molecules would equalize. Individually or in combination, these mechanisms would have to rely on the presence of a yet to be identified rate limiting factors or a combination of factors that constitute a more stable measure of centromere size. These would need to have a capacity to recognize chromatin-bound pool of CENP-A and contain ‘‘counting’’

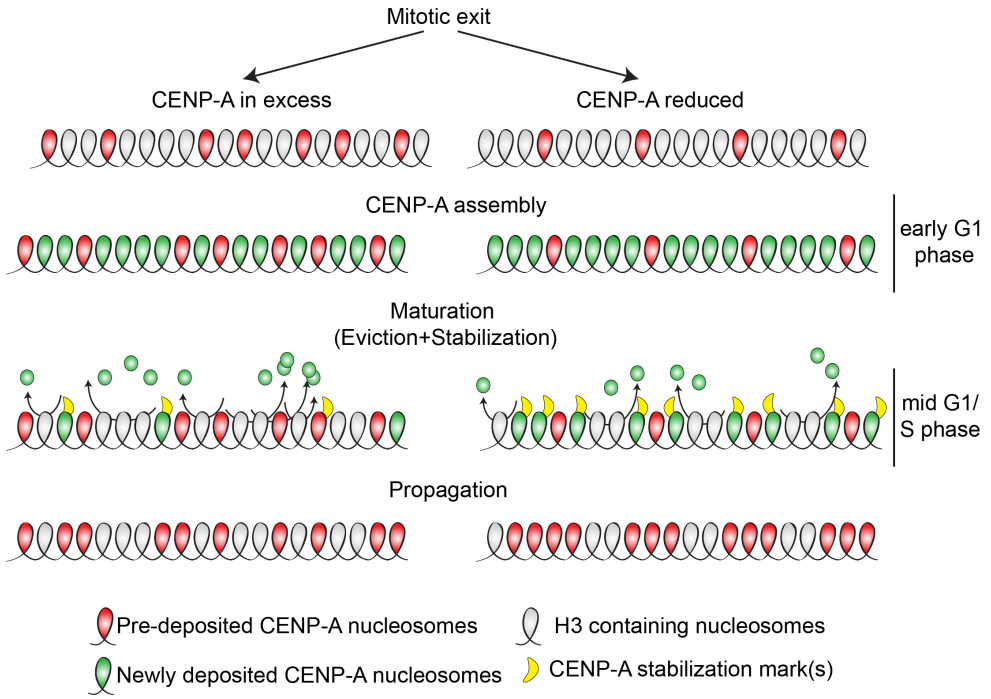

Figure 5.1 A model for normalization of CENP-A levels across mitotic divisions. Stochastic redistribution of CENP-A during S phase may give rise to daughter centromeres

Figure 5.1 Continued: having an unequal amount of parental nucleosomes upon mitotic exit. To accommodate for this, an excessive amount of nascent CENP-A is deposited to the centromere in early G1 phase, followed by selective stabilization of a portion of newly loaded CENP-A molecules. This would occur in an inverse proportion to the number of parental nucleosomes: the greater the number of parental nucleosomes is, the smaller the pool of new CENP-A is marked for stability, the remainder of which will be evicted. The combination of these two processes (stabilization and eviction) could encompass previously proposed ´´maturation´´ step of centromeric chromatin.

properties allowing sensing of the size of CENP-A populated domain. CENP-C, a factor stabilizing CENP-A (Falk et al., 2015) could be one of such factors, limiting CENP-A domain size.

5.4 Centromeric DNA, CENP-A and centromere

Current evidence point to an epigenetic mode of centromere establishment, maintenance and propagation (chapter 1 of this thesis). Alongside with experimentally induced *de novo* centromere formation achieved through artificial placement of CENP-A to naïve genomic loci (chapter 1), one of the strongest evidence for the epigenetic nature of the centromere are naturally occurring neocentromeres on DNA sequences unrelated to canonical centromeres (chapter 1). Consistently, length and content of centromeric DNA vary significantly even between closely related species. All of the above argues that DNA is neither necessary nor sufficient to drive centromere specification (chapter 1 of this thesis).

However, the majority of regional centromeres are associated with hundreds of kilobases of repetitive DNA satellites that can vary significantly between closely related species, arguing that a new centromeric repeat can sweep through the entire genome and rapidly replace the old repeat in all centromeres (Henikoff et al., 2001). Rapid evolution of centromeric DNA while the function of the centromere is conserved is known as ´´centromere

paradox´´. Additionally, centromere position can change over the course of evolution of a given species, as seen in comparative studies of chromosome structure in primates and other placental mammals, marsupials and birds (Carbone et al., 2006; Ferreri et al., 2005; Kasai et al., 2003; Ventura et al., 2001). A common feature of this ´´repositioned´´ or evolutionary new centromeres (ENCs) is the absence of any other significant and detectable change in markers order along the chromosome but the centromere position itself, arguing that a chromosome breakage-fusion cycles are not causative of ENC formation. Instead, it has been proposed that spontaneous inactivation of the ancestral centromere led to the formation of an epigenetically encoded neocentromere that had formed on non-repetitive DNA. Subsequently, these ´´young´´ centromeres gradually accumulate, over multiple successive generations, repetitive DNA through various recombination-based mechanisms, forming prevalent ´´mature´´ centromeres with repetitive centromeric DNA (Amor and Choo, 2002; Marshall et al., 2008; Montefalcone et al., 1999; Ventura et al., 2001). Therefore, this model predicts that many existing centromeres may have originated as neocentromeres. Along this line, different haplotypes associated with the same centromere in a population have been detected in potato (Gong et al., 2012; Wang et al., 2014). Five potato centromeres (Cen4, Cen6, Cen10, Cen11, and Cen12) assembled primarily on a single- or low-copy DNA sequences, thus structurally resembling neocentromeres. In contrast, six potato centromeres (Cen1, Cen2, Cen3, Cen5, Cen7, and Cen8) contained megabase-sized satellite repeat arrays all unique to individual centromeres. Similarly, centromeres of genus *Equus*, encompassing horses, donkeys and zebras, significantly shifted mitotically active centromere away from canonical α-satellite regions throughout their evolution (Piras et al., 2010). Importantly, some of the chromosomes

harbouring repeat-less centromeres still contained satellite DNA (without any mitotic function) whereas others contained no detectable repeats, indicating that part of the ENCs are result of a neocentromere activation on a non-repetitive DNA while others are likely the product of fusions between ancestral acrocentric chromosomes. Strikingly, CENP-A containing region of ENCs not only differs between different species, but also within the same species as well. Exploiting non-repetitive nature of horse centromere on chromosome 11 by combining CENP-A ChiP and Single Nucleotide Polymorphism (SNP), it was demonstrated that centromeric CENP-A is differentially binding distinct regions of non-repetitive DNA in the same individual, forming so called ´´positional alleles´´ (Purgato et al., 2014). The finding of centromere ´´sliding´´ on non-repetitive DNA was further exemplified by re-examination of experimentally derived chicken DT-40 neocentromeres (Hori et al., 2016). These neocentromeres were formed on various positions along the chromosome following excision of the endogenous centromere. Initially, upon examination of properties of various ´´freshly-made´´ neocentromeres, it was noted that they share remarkably similar CENP-A–associated regions. However, after prolonged expansion and clonal isolation in cell culture, the CENP-A-containing was found to diverge from the initially uniform one, forming discrete domains on a different positions compared to the original one, indicative of their shift along non-repetitive DNA. All combined, these results suggest that CENP-A domain can move along non-repetitive centromeric DNA.

One of the forces constraining the inherent mobility of CENP-A domains could be the repetitive nature of satellite DNA. Indeed, it has been proposed that the critical step in transition from ´´young´´ to ´´mature´´ centromere is acquisition of repetitive DNA because they probably confer an adaptive advantage possibly by increasing the accuracy of chromosome

segregation (Marshall et al., 2008). Whether it is hard to determine if ´´sliding´´ of CENP-A nucleosomes is also occurring on repetitive DNA, current evidence suggest that is not the case. Namely, human CENP-A nucleosomes tend to be highly phased on α-satellites, with a preferred positioning between functional CENP-B boxes (Hasson et al., 2013), which are the regions of α-satellite DNA to which CENP-B proteins binds (Earnshaw et al., 1987; Warburton, 2001). Intriguingly, the Y-chromosome centromere lacks functional CENP-B boxes yet CENP-A nucleosomes still show moderate phasing (albeit in lesser extent compared to CENB-B box containing), indicative of an intrinsic property of α-satellite to position CENP-A nucleosomes (Hasson et al., 2013). Therefore, centromeric α-satellites could be phasing or ``locking´´ CENP-A nucleosomes into their respective places, thus contributing to the prevention of CENP-A migration along the chromosome. Another contributory role of α-satellites, as proposed by (Marshall et al., 2008), is enhanced mitotic stability of ´´mature´´ centromeres compared to ´´young´´ ones. Indeed, even though neocentromeres are in principle mitotically stable, they lack competency to engage in efficient error correction of chromosome attachment to microtubules due to reduced and mislocalized AuroraB kinase, thereby jeopardizing faithful genome segregation (Bassett et al., 2010). Similarly, CENB-B box-less human Y chromosome displays an elevated frequency of erroneous mitotic segregation (Fachinetti et al., 2015). Consistently, even though binding of CENP-B binding to CENP-B boxes is not necessary for survival in absolute terms (as exemplified by viable CENP-B knockout mice (Hudson et al., 1998)), the presence of CENP-B enhances mitotic fidelity of α-satellites containing centromeres through stabilization of CENP-C on one hand and kinetochore formation on the other (Fachinetti et al., 2015; Hoffmann et al., 2016). Based on this, the emerging model of centromere

evolution involves initial specification of the centromere genomic location by epigenetic mechanism governed by CENP-A (´´young´´ centromere), followed by subsequent selection and stabilization of centromeric domain by invading repetitive DNA elements (´´mature´´ centromere). However, the association of the ´´mature´´ centromere with repetitive DNA has an evolutionary cost, reflected by the highly recombinogenic nature of these repeats that can lead to their expansion in size across multiple generation (Malik and Henikoff, 2009). This expansion can in turn lead to the formation of a bigger centromere domain which could have profound consequences during asymmetric female meiosis, in a process termed ´´centromere drive´´ or ´´selfish´´ centromere hypothesis (Kanizay and Dawe, 2009; Malik and Henikoff, 2002). Centromere drive is a variant of the meiotic drive hypothesis, in which certain ´´selfish´´ genomic elements have a transmission advantage in female meiosis, thus skewing Mendelian ratios of inheritance. According to the centromere-drive hypothesis, centromeric DNA acts as a selfish genetic element whereby a bigger centromeric DNA domain will assemble a larger (stronger) centromere domain, which in turn will nucleate a bigger kinetochore attracting greater number of microtubules, thus exploiting asymmetric female meiosis to promote its preferential transmission to the egg. This retention can lead to increased frequency of one chromosome compared to its homolog, allowing it to sweep through a population along with possibly deleterious hitchhiking mutations. In female meiosis, the centromere doesn't have any cost for fertility. However, in male meiosis an imbalance in centromere strength could lead to increased nondisjunction and obstruction of meiosis, resulting in either reduced fertility or sterility. To counteract these deleterious effects, compensatory or suppressing mutations are expected to arise, particularly in the basic unit of centromere inheritance, the CENP-A molecule. These

putative mutations would be modulating the affinity of CENP-A to bind or recognize changed satellite DNA, thus suppressing the expansion of centromere domain (Henikoff et al., 2001). Indeed, most evolutionary changes in the sequence of CENP-A occur in the N terminus and loop 1 of CENP-A, which is directly contact DNA (Black et al., 2004; Malik et al., 2002). Additionally, the CENP-A specific chaperone-HJURP and CENP-A are co-evolving whereby changes in loop1 of CENP-A are accompanied by complementary changes in the chaperone, which ultimately determines perpetuation of centromeric domain (Rosin and Mellone, 2016). Therefore centromeric DNA, CENP-A and its loading machinery, are rapidly evolving due to the ongoing evolutionary conflict in which repetitive DNA continually attempts to hijack meiotic machinery to its own benefit, and its suppressing force manifested in invention of lineage specific centromeric proteins.

5.5 Seeding the centromere

The process of neocentromere activation argues that the overall genome has the capacity, in principal, to nucleate a functional centromere. Indeed, even in the presence of an active centromere, the non-centromeric pool of CENP-A is scattered around the genome in a surprisingly elevated amounts (Bodor et al., 2014). Could this pool of CENP-A serve as a priming template for seeding of a new centromere? Experimentally induced neocentromeres in *C. albicans* and chicken DT40 cells tend to form in the proximity of the old one, due to the fact that even though the occupancy of CENP-A is at its highest at the centromeres, a significant amount trails to either side of it, forming a platform for neocentromere formation (Ketel et al., 2009; Shang et al., 2013; Thakur and Sanyal, 2013). Additionally, chicken neocentromeres formed at both transcriptionally active and inactive chromosome loci, showing no preference for DNA sequence (Shang et al.,

2013). Interestingly, formation of neocentromeres is inversely correlated with transcriptional activity of a given locus; high transcriptional activity seems incompatible with neocentromere formation whereas in cases when neocentromere is placed on an active gene, gene expression is significantly reduced. In. *S. pombe* neocentromeres formed infrequently proximal to the excised endogenous centromere, likely due to the removal of both CENP-A containing domain and pericentric chromatin (Ishii et al., 2008). Neocentromeres in this case clustered at the heterochromatic subtelomeric region. Similarly, in Drosophila, genomic regions near or within heterochromatin are preferred sites of neocentromere formation (Heun et al., 2006; Ketel et al., 2009b; Mendiburo et al., 2011; Olszak et al., 2011). Consistently, in fission yeast, regions depleted of H2A.Z histone variant, which tend to be heterochromatic, are more permissive for neocentromere formation (Ogiyama et al., 2013). Therefore, although DNA sequence *per se* doesn't specify centromere formation, certain favorable genomic or chromatin can enhance centromere seeding.

Given that CENP-A itself is sufficient to nucleate a functional centromere, coupled with the propensity of CENP-A domains to migrate along non-repetitive DNA along with the permissiveness of the genome for centromere nucleation, the critical question is how, once nucleated, the centromere is faithfully maintained and propagated throughout multiple cell divisions? Certainly, stabilizing effects of repetitive DNA can contribute to overall maintenance of stable centromeric domain (as discussed previously). However, on an evolutionary time-scale association of the CENP-A domain with repetitive DNA would happen in a gradual fashion, indicating that other factors may play a principal role in maintaining an active centromere. CENP-A, as many other histones, is deposited and incorporated into nucleosomes by its dedicated chaperone HJURP. CENP-

A and HJURP exist in a soluble, prenucleosomal complex throughout the cell cycle (chapter 3 of this thesis). Interestingly, human HJURP can recognize and bind DNA through its middle region (271-386aa), and this interaction is proposed to be vital for centromeric CENP-A incorporation (Müller et al., 2014). An evolutionary process can be envisioned in which HJURP ´´scouts´´ sites across the genome which would allow centromere seeding (Figure 5.2). This process may depend on the low levels of non-centromeric CENP-A nucleosomes dispersed across the genome. Once a seeding event has established the minimal CENP-A domain, (here named ´´immature centromere``), the survival of the centromere will dependent on the combinatorial effect of (a) permissive chromatin environment and (b) capacity of CENP-A nucleosomes to initiate a positive epigenetic feedback loop. This feed-forward loop would ultimately rely on CENP-A levels surpassing the critical threshold (reaching critical density) making them a distinguishable feature compared to the rest of the genome. Therefore, ample amount of initial CENP-A would be followed by recruitment of more HJURP/CENP-A complex and possibly some other centromere components (such is CENP-C), thus further stabilizing the ´´immature`` centromere. If indeed HJURP scans the genome for an adequate place for CENP-A deposition, this is probably occurring in a stochastic manner, raising the possibility of multiple seeding events on a single chromosome (´´single step establishment``, Figure 5.2). Therefore, a scenario may occur in which an ´´immature´´ centromere competes with the newly seeded ones, while the differences in the amount of CENP-A present at either could be rather minor. One possible solution overcoming this problem could be the postulation of an additional factor that would in a highly-specific manner recognize chromatin-bound CENP-A nucleosomes (´´two-step establishment``, Figure 5.2). Importantly, this putative factor would have to

´´sense´´ small differences in the size of CENP-A bound domain (either directly or indirectly) therefore distinguishing the ´´immature`` centromere from the competing ones. Accordingly, this ``reader´´ of CENP-A domain will be targeted to chromatin in a CENP-A-density-dependent manner whereby more CENP-A would direct a higher level of ´´reader`` chromatin recruitment and vice versa (Figure 5.2, right). Additionally, the ´´reader´´ of the CENP-A domain could be present in limiting amount, such that there is only enough for one centromere. In this scenario, the centromere having the highest amount of CENP-A nucleosomes would sequester this factor away from the competing centromeres. Alternatively, this hypothetical factor could be operating in ´´all or nothing´´ fashion by not recognizing the low amount of CENP-A nucleosomes which have not exceeded a critical threshold. In either case, the presence of this factor would additionally amplify the initial CENP-A seeding signal, further stabilizing the formation of what would become a “mature”` centromere. One of the candidates to perform this function is the CENP-A ´´licencing´´ factor, the M18 complex, whose centromere targeting is necessary for perpetuating centromeric chromatin in a myriad of species (chapter 2 of this thesis). Moreover, HJURP centromeric targeting is dependent on Mis18 centromeric localization, consistent with a ´´reader`` function of the M18 complex. A second and complementary aspect of the centromere competition model could be limiting CENP-A chromatin incorporation to a discrete window of time. Indeed, CENP-A propagation is tightly coupled to cell cycle progression (Jansen et al., 2007; Silva et al., 2012; Stankovic et al., 2017). Assuming that the rate of CENP-A chromatin incorporation is dictated by the amount of CENP-A itself (Bodor et al., 2014) and is driven by two factors: the CENP-A domain ´´reader`` (the M18 complex?) and HJURP, a slight difference in the amount of CENP-A residing at the ´´immature´´ and

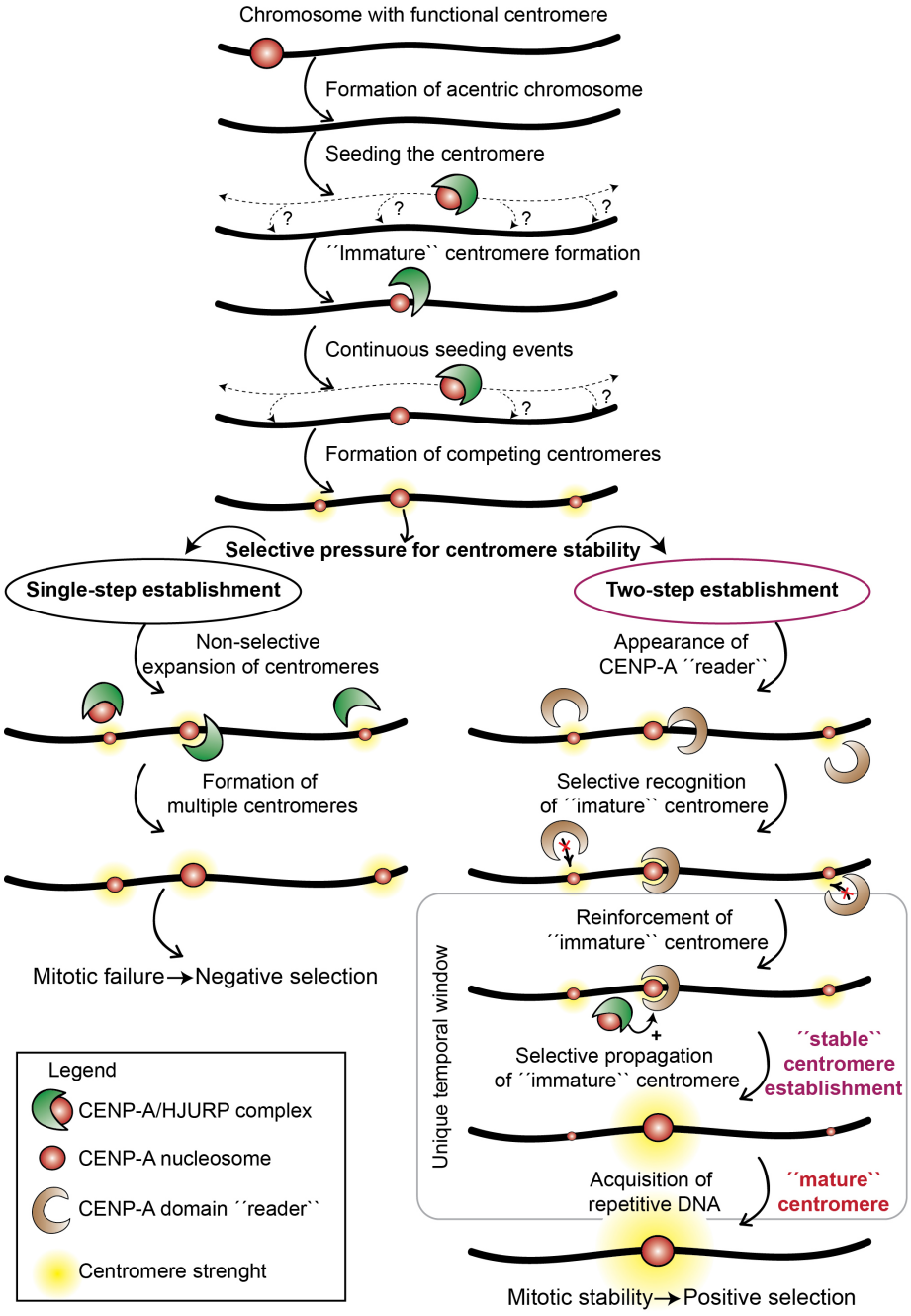

Figure 5.2 Evolutionary mechanisms counteracting formation of multiple centromeres. Following inactivation of ancestral centromere (spontaneous centromere

Figure 5.2 Continued: silencing, chromosome breakage) the acrocentric chromosome is under a selective pressure to form a functional centromere. Within the prenucleosomal CENP-A complex, HJURP ´´scans`` permissive genomic sites for nascent CENP-A incorporation in a stochastic manner. This process might be facilitated by low-level of genomic CENP-A. Initial seeding event gives rise to ´´immature´´ centromeres. Continuation of HJURP chromatin targeting/CENP-A incorporation cycles results in formation of deleterious multi-centric chromosome, which is removed from the population (single-step establishment). Alternatively, appearance of a factor that specifically recognizes nucleosomal CENP-A in dose-dependent-manner, may limit HJURP activity to a specific genomic locus. Coupled with temporal control of ``reader´´ and HJURP chromatin targeting, the ´´immature´´ centromere, over time, accumulates copious amount of CENP-A, allowing it to initiate a positive epigenetic feedback loop and transition into ´´stable´´ centromere (two-step establishment). Subsequent acquisition of repetitive DNA ensues development of ´´mature`` centromere.

competing centromeres would be further amplified, allowing the ´´immature´´ centromere to outcompete other nascent CENP-A domains and transition into a ´´stable´´ centromere. The combinatorial effects of a two-factor driven, faithful reinforcement of the CENP-A domain, coupled with a temporally controlled equilibration of CENP-A levels, provide a strong basis for a robust epigenetic inheritance of the ´´stable´´ centromere (Figure 5.2, right). Once established, the ´´stable´´ centromere may opt to maintain these mechanisms to drive its own mitotic propagation, and, together with invasion of repetitive DNA evolve into ´´mature`` centromere.

Although highly speculative, the centromere competition model provides an evolutionary framework for understanding the forces driving establishment and propagation of the epigenetic centromere, whereby mechanism that counteract formation of deleterious multiple centromeres have been integrated into a common pathway that drive faithful mitotic centromere inheritance. The two-step inhibitory pathway that is limiting CENP-A incorporation to a unique cell cycle window, described in chapter 4 of this

thesis, may represent an evolutionary solution ensuing maintenance and propagation of epigenetic centromere.

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Appendix 1

Reductionism at the vertebrate kinetochore

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Reductionism at the vertebrate kinetochore

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The kinetochore forms the site of attachment for mitotic spindle microtubules driving chromosome segregation. The interdependent protein interactions in this large structure have made it difficult to dissect the function of its components. In this issue, Hori et al. (2013. *J. Cell Biol.* http://dx.doi.org/10.1083/jcb.201210106) present a novel and powerful methodology to address the sufficiency of individual proteins for the creation of a functional de novo centromere.

The centromere is the chromosomal locus responsible for kinetochore nucleation and chromosome segregation in mitosis (Cheeseman and Desai, 2008). In most organisms, centromere position is specified epigenetically through a unique chromatin structure marked by the presence of the histone H3 variant CENP-A and a constitutive complex of centromere proteins (Foltz et al., 2006; Okada et al., 2006). CENP-A is a particularly promising candidate for the epigenetic marking of the centromere, as targeted deposition of CENP-A has been shown to lead to the formation of a functional kinetochore, which can be heritably maintained (Barnhart et al., 2011; Guse et al., 2011; Mendiburo et al., 2011). How CENP-A is recruited to the centromere site and how the associated centromere complex defines the kinetochore are key current questions.

Understanding the kinetochore puzzle

The constitutive centromere-associated network (CCAN) of 16 proteins is thought to form a functional bridge, linking centromeric CENP-A chromatin to the kinetochore. In a study reported in this issue, T. Fukagawa and his team dissect the direct contribution of different CCAN components to the nucleation of centromeric chromatin as well as to recruitment of the microtubule-binding complex of the outer kinetochore. They expand on a methodology recently used by the same group in collaboration with I. Cheeseman's laboratory. In that study, truncated versions of CENP-C and CENP-T, two proteins that are part of the CCAN, were fused to the Lac repressor protein (LacI) and tethered to chromosomally integrated arrays of bacterial Lac operator (LacO) sequences (Gascoigne et al., 2011). These engineered chromosomal foci consisting of both CENP-C and CENP-T led to recruitment of the outer kinetochore and generated transient functional microtubule attachments.

In the study in this issue, Hori et al. take this approach a major step further. Now, the authors combined the LacI–LacO tethering system with conditional deletion of an endogenous, loxP-flanked centromere, which they previously developed (Fig. 1 A; Shang et al., 2010). By deleting the centromere of the only Z chromosome in chicken DT40 cells, the authors were able to test directly which LacI fusion proteins can rescue chromosome segregation and viability. In human cells, de novo centromere formation has been promoted on ectopically introduced alphoid DNA arrays to which CENP-A deposition factors were tethered (Ohzeki et al., 2012). A strategy to build artificial kinetochores on whole chromosomes has previously been achieved in yeast (Kiermaier et al., 2009; Lacefield et al., 2009) but had thus far not seen its counterpart in a vertebrate cell system.

Strikingly, targeting of the CCAN components CENP-C, CENP-I, or the CENP-A–specific chaperone HJURP is sufficient to initiate a heritable centromere at the LacO array. This ectopic centromere functionally replaced the endogenous one and faithfully maintained chromosome Z ploidy in the cell population. Consistently, the NDC80 complex, the principal microtubule-binding module, and other components of the kinetochore known as the KMN (KNL-1/Mis12/Ndc80) network (Cheeseman et al., 2006) were corecruited to the ectopic site, as well as the chromosomal passenger complex (CPC), components at the inner centromere. Remarkably, CENP-A centromeric chromatin was assembled not only after deposition of its chaperone HJURP but also after tethering of CENP-C or CENP-I (Fig. 1 B). Although these two CCAN components have been shown to be required for CENP-A assembly at endogenous centromeres (Okada et al., 2006; Erhardt et al., 2008; Carroll et al., 2010), these results now show they can also be sufficient for de novo recruitment of CENP-A on naive chromatin. Importantly, this implies that although CENP-A chromatin provides a stable heritable core, its propagation involves a positive epigenetic feedback mechanism in which other CCAN components, themselves dependent on CENP-A, play an active role in CENP-A recruitment.

Building a minimal kinetochore

LacI-mediated targeting of CENP-T or the N terminus of CENP-C, both of which make contacts to the outer kinetochore, proved also sufficient to generate functional ectopic kinetochores. Importantly, analysis of their architecture revealed that these lacked CCAN components, including CENP-C and CENP-A, but recruited the CPC components, providing a functional link between the centromere complex and the inner centromere (Fig. 1 B).

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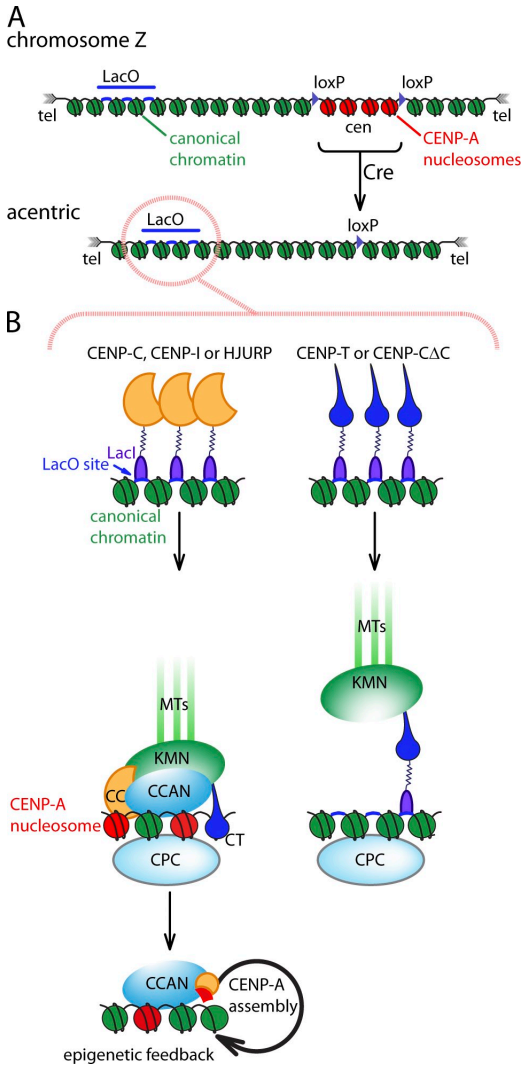

Figure 1. **Engineering vertebrate centromeres.** (A) The endogenous centromere from DT40 chromosome Z is deleted by Cre-loxP–mediated excision. The chromosome is engineered to carry an array of LacO sites. tel, telomere. (B) Fusion of CENP-C, CENP-I, or the CENP-A chaperone HJURP to the Lac repressor (LacI) tethers these proteins to the LacO array and leads to functional replacement of the endogenous centromere through recruitment of centromeric chromatin (CENP-A) and centromere complex (CCAN), the inner centromere (CPC), and the kinetochore (KMN). The CCAN factors CENP-C (CC) and CENP-I are sufficient for CENP-A chromatin establishment, indicating they play a direct role in the maintenance of a heritable centromere core. Tethering of CENP-T (CT) or the CENP-C N terminus (CENP-CΔC) leads to functional LacI tether-dependent kinetochore formation through recruitment of KMN components and the CPC but lacking the remainder of the CCAN. MTs, microtubules.

Despite the lack of centromeric chromatin, these ectopic kinetochores maintained chromosome Z segregation, although not quite as efficiently as a full-fledged neocentromere carrying the remainder of the CCAN and CENP-A chromatin. Consequently, these kinetochores are continuously dependent on the LacI–LacO interaction, as allosteric disruption of LacI binding with IPTG led to rapid loss of chromosome Z. In contrast, CENP-A chromatin/CCAN-containing neocentromeres are independent of the initial seeding event and, once formed, can be weaned from LacI.

These results provide an important functional insight in the role of the centromere complex. On the one hand, it specifies the site of recruitment of kinetochore proteins, whereas on the other, it forms an integral component of a heritable self-replicating protein complex that provides a stable chromosomal anchor. Finally, the system developed by Hori et al. (2013) offers exciting prospects. The construction of highly simplified vertebrate artificial chromosomes in comparison to those currently available, which typically rely on the use of large arrays of centromere-associated DNA repeats, will likely help the field answer critical questions ahead.

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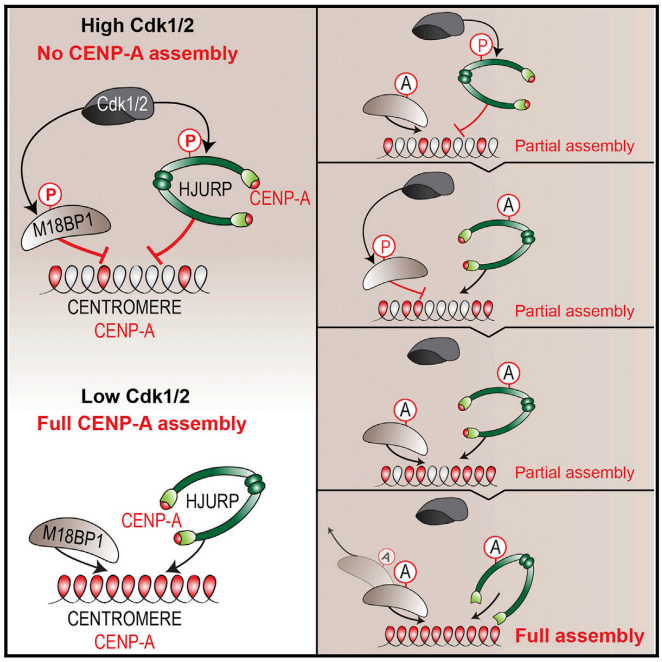
Appendix 2

A Dual Inhibitory Mechanism Sufficient to Maintain Cell-Cycle-Restricted CENP-A Assembly

Molecular Cell

A Dual Inhibitory Mechanism Sufficient to Maintain Cell-Cycle-Restricted CENP-A Assembly

Graphical Abstract



Highlights

- The HJURP conserved domain interacts with cyclin A
- Timing of M18BP1 localization is controlled by T653 phosphorylation
- Control of HJURP and M18BP1 suffices to cell-cycle restrict CENP-A assembly
- CENP-A assembly results in functional removal of M18BP1 from centromeres

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In Brief

Centromeres are epigenetically defined by the heritable histone H3 variant CENP-A, whose assembly is restricted to G1 phase. Stankovic et al. show that cell-cycle coupling is achieved through inhibitory Cdk-mediated phosphorylation of two key CENP-A assembly factors, M18BP1 and HJURP, preventing unscheduled centromeric chromatin assembly outside of the G1 phase.

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A Dual Inhibitory Mechanism Sufficient to Maintain Cell-Cycle-Restricted CENP-A Assembly

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SUMMARY

Chromatin featuring the H3 variant CENP-A at the centromere is critical for its mitotic function and epigenetic maintenance. Assembly of centromeric chromatin is restricted to G1 phase through inhibitory action of Cdk1/2 kinases in other phases of the cell cycle. Here, we identify the two key targets sufficient to maintain cell-cycle control of CENP-A assembly. We uncovered a single phosphorylation site in the licensing factor M18BP1 and a cyclin A binding site in the CENP-A chaperone, HJURP, that mediated specific inhibitory phosphorylation. Simultaneous expression of mutant proteins lacking these residues results in complete uncoupling from the cell cycle. Consequently, CENP-A assembly is fully recapitulated under high Cdk activities, indistinguishable from G1 assembly. We find that Cdk-mediated inhibition is exerted by sequestering active factors away from the centromere. Finally, we show that displacement of M18BP1 from the centromere is critical for the assembly mechanism of CENP-A.

INTRODUCTION

Centromeres are chromosomal loci that drive faithful genome segregation during mitotic division (Allshire and Karpen, 2008). The functional foundation of the centromere is established by a specialized chromatin structure that features the histone H3 variant CENP-A (Black and Cleveland, 2011). This CENP-A-based chromatin domain provides a structural platform for formation of the kinetochore, which links chromosomes to spindle microtubules during mitosis (Cheeseman and Desai, 2008; Foltz et al., 2006; Okada et al., 2006). In addition, CENP-A ensures stable maintenance of the centromere position through an epigenetic self-propagating feedback loop (Black and Cleveland, 2011; Gómez-Rodríguez and Jansen, 2013). Support for the epigenetic nature of the centromere comes from naturally occurring neocentromeres (Amor et al., 2004; Marshall et al., 2008), where centromere proteins vacate the original centromeric DNA sequence and assemble heritably on previously naive chromatin. In addition, ectopic targeting of CENP-A or proteins of the centromere complex to a non-centromeric locus was shown to be sufficient to initiate a functional and heritable centromere (Barnhart et al., 2011; Hori et al., 2013; Mendiburo et al., 2011). Consistent with a key role at the core of a positive epigenetic feedback loop, CENP-A nucleosomes are long lived and are maintained through multiple cell divisions (Bodor et al., 2013; Jansen et al., 2007). The unusually slow turnover of CENP-A at each centromere (Falk et al., 2015) indicates that replenishment is either equally slow or limited in time and tied to CENP-A redistribution following DNA replication. Indeed, in metazoans, assembly of newly synthesized CENP-A is directly linked to cell-cycle progression and is initiated during mitotic exit and restricted to the early G1 phase of the cell cycle (Jansen et al., 2007; Schuh et al., 2007).

Previously, we showed that brief inhibition of cyclin-dependent kinase 1 and 2 (Cdk1/2) activities is sufficient to drive CENP-A deposition prior to mitotic exit (Silva et al., 2012). This has led to a model where the CENP-A assembly machinery is present and poised for activity but is kept inactive throughout S, G2, and M phase until mitotic exit, when activities of Cdk1/2 drop, concomitant with the onset of CENP-A deposition. Key proteins necessary for the process of CENP-A deposition include the Mis18 complex and the CENP-A chaperone HJURP, which bears CENP-A-specific nucleosome assembly activity (Dunleavy et al., 2009; Foltz et al., 2009; Fujita et al., 2007). HJURP and M18BP1 (also known as HsKNL2), a member of the Mis18 complex, are phosphoproteins (Bailey et al., 2016; Dephoure et al., 2008; Kato et al., 2007; McKinley and Cheeseman, 2014; Müller et al., 2014; Silva et al., 2012; Wang et al., 2014) and localize to centromeres in a cell-cycle-controlled manner in the early G1 phase (Dunleavy et al., 2009; Foltz et al., 2009; Fujita et al., 2007; Maddox et al., 2007), indicating they are putative

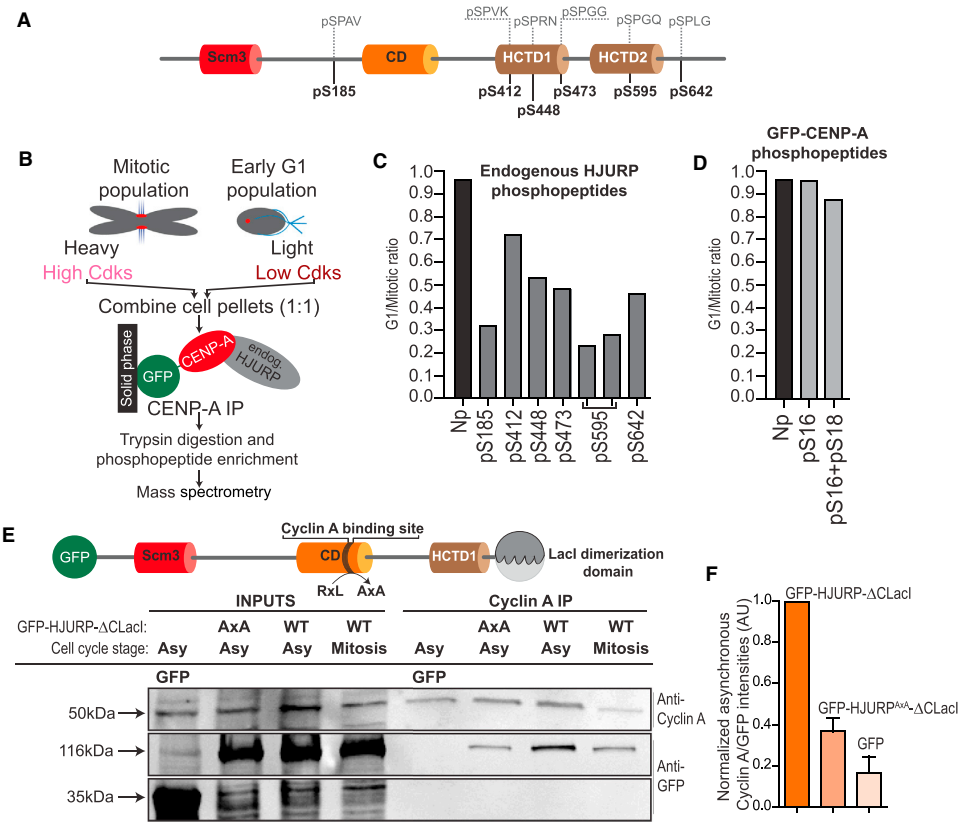

Figure 1. HJURP Is Phosphorylated in a Cdk-Dependent Manner and Interacts with Cyclin A

(A) Schematic representation of HJURP protein (Scm3, CENP-A binding domain; CD, conserved domain; HCTD, HJURP C-terminal domain). Position of phospho-sites identified by SILAC in (C) are indicated. Amino acid sequences flanking phospho-sites are annotated in gray.

(B) Schematic of SILAC experiment (see Supplemental Experimental Procedures for details). Light cells were released into G1 by Roscovitine treatment for 30 min. At this stage, HJURP is partially dephosphorylated (see Figures S1F and S1G).

(C) The L/H ratios of phosphorylated Cdk sites detected on endogenous HJURP are listed. A representative non-phosphorylated peptide (Np) is shown as internal control. Note: pS595 was detected on two independent peptides.

(D) L/H ratios of Cdk consensus sites within the N-terminal tail of CENP-A (see Figure S2 for data from two additional replicate experiments).

(E) HJURP CD mediates interaction with cyclin A. Top: schematic representation of HJURP protein. The mutation of conserved RxL motif to AxA is annotated with a black arrow. Experiments are performed with an HJURP construct in which the C-terminal homodimerization domain is replaced with that of LacI to prevent dimerization with wild-type HJURP. Bottom: co-immunoprecipitation (IP) of extracts expressing indicated constructs either from asynchronous or mitotically enriched cells. Bound complexes were separated using SDS-PAGE, followed by immunoblotting with indicated antibodies.

(F) Quantification of IP experiments. The GFP signal from each IP was normalized to corresponding cyclin A signal and input GFP signal in order to control for IP efficiency and GFP fusion protein expression level, respectively. GFP-HJURP signals were set to 1. Error bars indicate SEM from three independent experiments. See also Figures S1 and S2.

targets for Cdk regulation. In addition, recent work has identified the mitotic kinase Plk1 as a critical component to drive CENP-A assembly (McKinley and Cheeseman, 2014). However, while Plk1 is itself a cell-cycle-controlled kinase, it does not restrict CENP-A assembly to the G1 phase, because it is required for both canonical assembly in G1 phase as well as premature assembly upon Cdk inhibition. In addition, several residues on CENP-A itself are phosphorylated (Bailey et al., 2013; Yu et al., 2015; Zeitlin et al., 2001). One of these, serine 68, is proposed to be phosphorylated by mitotic Cdk activity (Yu et al., 2015), but the relevance of this is being disputed (Fachinetti et al., 2017), and mutation of this residue does not lead to a change in the timing of CENP-A deposition. In contrast, mutations of phospho-residues in HJURP or artificial recruitment of M18α to centromeres has been reported to result in premature centromere recruitment of CENP-A (McKinley and Cheeseman, 2014; Müller et al., 2014). While these studies point to a contributing role for these factors, they leave open the critical questions of

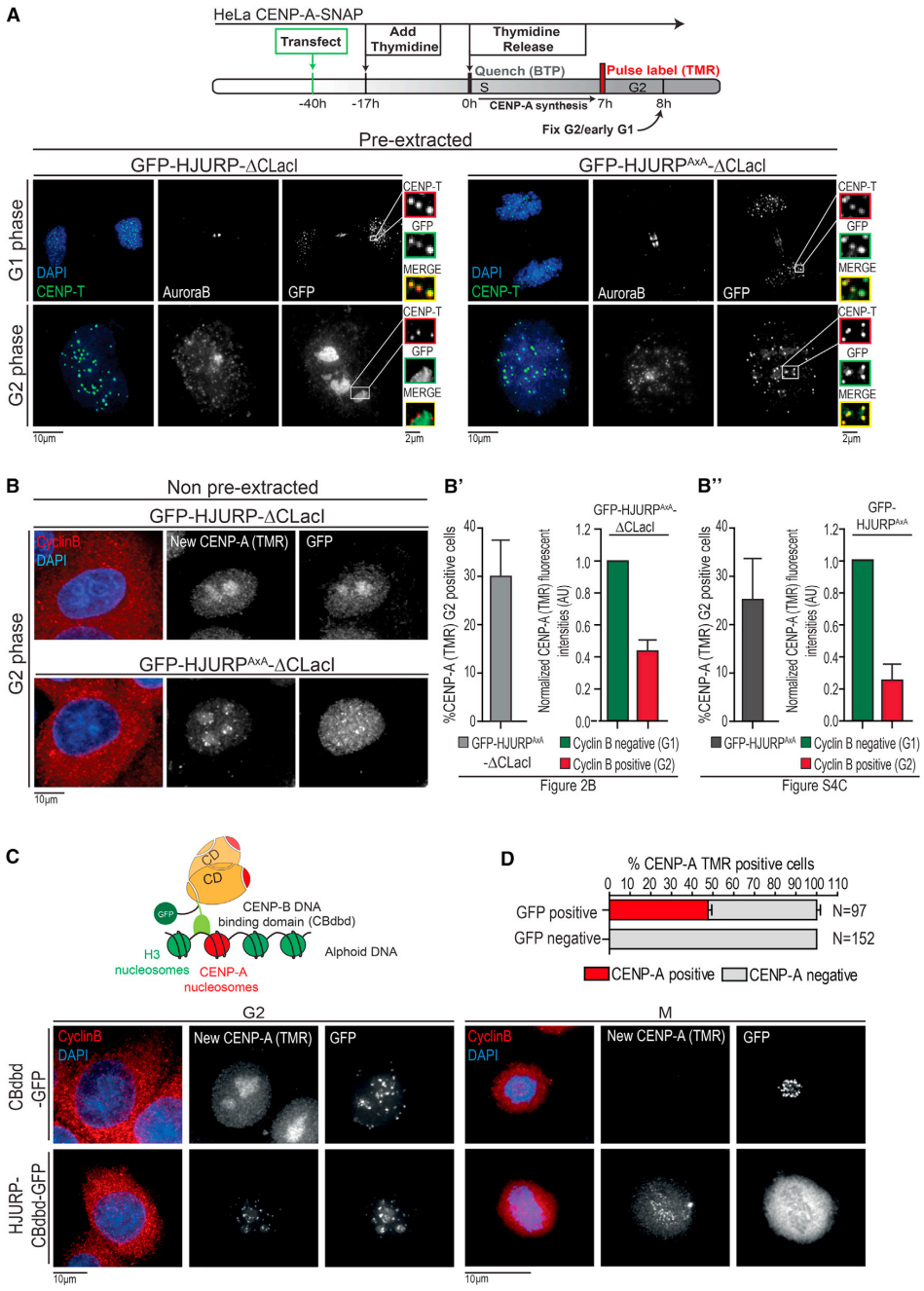

Figure 2. Timing of HJURP Targeting and CENP-A Deposition Is Controlled by HJURP CD

(A) HeLa CENP-A-SNAP cells were transiently transfected with indicated constructs, and thymidine synchronized to enrich cells in the G2 phase. Cells were permeabilized prior to fixation and counterstained for Aurora B, CENP-T, and DAPI to distinguish between G2 and early G1 cell-cycle phases, centromere localization, and DNA, respectively. GFP booster was used to amplify the GFP-HJURP fluorescent signal.

(legend continued on next page)

which factors are necessary, which are sufficient, how Cdk-mediated control is exerted, and how key proteins are functionally inhibited.

To resolve the specific molecular steps that ensure cell-cycle-restricted CENP-A assembly, we report full uncoupling of CENP-A assembly from the cell cycle and Cdk regulation. To achieve this, we identified a functional cyclin-interacting domain in HJURP and a critical phospho-site in M18BP1. Simultaneous uncoupling of these factors from cell-cycle progression results in a complete premature reconstitution of CENP-A assembly process in the G2 phase prior to mitotic exit. Our results identify a dual inhibitory mechanism that is sufficient to maintain cell-cycle-restricted centromere propagation and define the molecular underpinnings of how assembly is turned on and subsequently turned off.

RESULTS

HJURP Is Phosphorylated in a Cell-Cycle-Dependent Manner

HJURP, the CENP-A-specific chaperone, is a phospho-protein and features several putative Cdk sites (Figure 1A; Bailey et al., 2016; Dephoure et al., 2008; Kato et al., 2007; Müller et al., 2014; Wang et al., 2014), making it a prime candidate for cell-cycle control of CENP-A assembly. To quantitatively measure HJURP phosphorylation, we used stable isotope labeling by amino acids in cell culture (SILAC) coupled to mass spectrometry. This allowed us, in an unbiased manner, to precisely determine which residues are phosphorylated under high Cdk conditions and how they respond to changes in Cdk activity. Cdk1 levels differ most dramatically between mitosis and the G1 phase. We therefore compared levels of phospho-peptides on the prenucleosomal GFP-CENP-A/HJURP complex between populations of mitotically arrested cells and cells that were released from mitotic arrest by Roscovitine-mediated Cdk inhibition (Figure 1B). Normal timing and efficiency of CENP-A assembly was preserved under these conditions (Figure S1). We detected six phosphorylated residues corresponding to putative Cdk consensus sites within HJURP, all of which were dephosphorylated upon mitotic exit, ranging from a 25%–70% decrease relative to mitotic values (Figures 1C and S2). Although three of these sites (S412, S448, S473) correspond to reported phospho-sites (Müller et al., 2014; Wang et al., 2014), our analysis shows that these are neither the sole nor the most responsive sites to inactivation of Cdks, at least in mitosis. In contrast, no change was observed at unphosphorylated peptides of HJURP (Figure 1C) or at Cdk-consensus phospho-sites on the CENP-A N-terminal tail (Bailey et al., 2013) after forced mitotic exit (Figure 1D), indicating that protein levels of CENP-A and HJURP remain unaffected (see also Figures S1F and S1G) and that HJURP is selectively dephosphorylated.

The HJURP-Conserved Domain Interacts with Cyclin A and Controls the Timing of CENP-A Assembly

Our findings from SILAC experiments led us to focus on HJURP in particular and determine how its phospho-regulation is coupled to the control of cell-cycle timing of CENP-A chromatin assembly. Although the canonical consensus site for Cdks is (S/T)PX(K/R) (Hagopian et al., 2001; Holmes and Solomon, 1996), five of the six phospho-sites in HJURP that are affected by Cdk inactivation display a shorter (S/T)P motif (Figure 1A) (Errico et al., 2010). Phosphorylation of such truncated motifs often requires additional cyclin-binding sites for enhanced substrate recognition (Adams et al., 1996; Russo et al., 1996). Indeed, we found a typical cyclin A-binding RxL motif (Brown et al., 2007) within a vertebrate conserved domain (CD) of HJURP, which had no previously described function (Sanchez-Pulido et al., 2009). We tested whether HJURP interacts with cyclin A and B, the major drivers of Cdk activity in S/G2 phase and mitosis, respectively, all stages at which CENP-A assembly is inhibited (Silva et al., 2012). We performed either cyclin B or cyclin A co-immunoprecipitation from HEK293T cells in which we ectopically expressed either GFP-tagged HJURP with a mutated RxL motif (RLL > ALA, henceforth referred to as HJURP^{AxA}), or with a wild-type CD. HJURP forms a homodimer (Zasadzińska et al., 2013). To avoid cross-dimerization with endogenous HJURP, we replaced its C-terminal domain with that of LacI (henceforth named HJURP-ΔCLacI), which does not interfere with the CENP-A chaperoning and assembly activity of HJURP, as described by Zasadzińska et al., 2013. Cyclin A robustly co-immunoprecipitated GFP-tagged HJURP-ΔCLacI (Figures 1E and S3A). In contrast, GFP-HJURP^{AxA}-ΔCLacI pull-down was reduced by 70% compared to HJURP-ΔCLacI, carrying a wild-type CD (Figure 1F). Mitotically enriched cells (low cyclin A) were used as a control to demonstrate that HJURP pull-down is cyclin A dependent. Consistent with the fact that inhibition of CENP-A assembly is maintained in mitosis (Jansen et al., 2007), even though cyclin A is degraded in early mitosis (den Elzen and Pines, 2001; Geley

(B) Experiments were performed as in Figure 2A, except here, CENP-A assembly was assayed using SNAP TMR-labeling of its S-phase-synthesized pool. Following fixation, cells were counterstained for cyclin B and DAPI to indicate G2 status and DNA, respectively. (See Figure S4C for extended analysis of GFP-HJURP- and GFP-HJURP^{AxA}-induced assembly.)

(B′) Left: quantification of frequency of premature CENP-A loading in cyclin B-positive cells expressing GFP-HJURP^{AxA}-ΔCLacI. Right: quantification of CENP-A-SNAP (TMR) fluorescent signal intensities of cells from the experiment on the left in the G2 phase (cyclin B positive) and the G1 phase (cyclin B negative) using CENP-T signal as a centromere reference (not depicted). Centromeric CENP-A-SNAP fluorescent signals were normalized to the average of G1 cell signals in each experiment (not considering the difference in replicated sister G2 centromeres versus segregated G1 centromeres). Three replicates, error bars indicate SEM.

(B″) Left: quantification of frequency of premature CENP-A loading in cyclin B-positive cells expressing GFP-HJURP^{AxA} from three replicate experiments (see Figure S4C for images). Right: quantification of CENP-A-SNAP (TMR) fluorescent signal intensities from the same experiment.

(C) Top: schematic of relevant domains in centromere-targeted HJURP. Bottom: HeLa CENP-A-SNAP cells were transfected with indicated constructs. 7 hr post thymidine release, cells were either fixed in the G2 phase or collected in nocodazole to enrich for mitotic cells. Cells were counterstained for cyclin B and DAPI to indicate G2 status and DNA, respectively.

(D) Quantification of frequency of premature CENP-A-SNAP deposition in cyclin B-positive cells driven by expression of Cbdb-HJURP-GFP. Error bars indicate SEM. See also Figure S3 and S4.

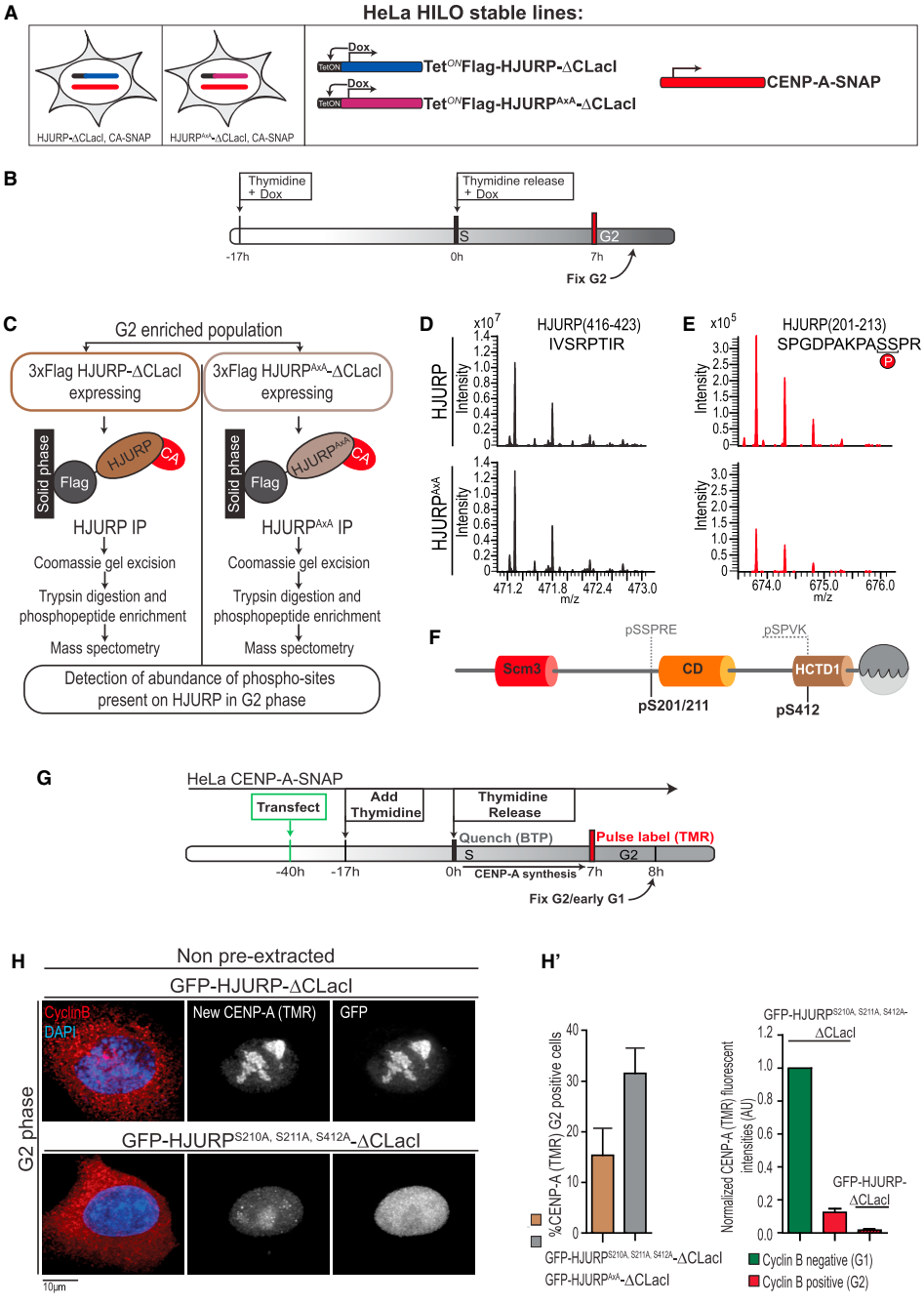

Figure 3. HJURP Serine 210/211 Is Functionally Phosphorylated in G2 Phase Cells

(A) Schematic of cell lines used for a label-free mass spectrometry analysis.

(B) HeLa HILO cells carrying indicated doxycycline-inducible HJURP constructs were enriched in G2 cells by thymidine arrest and release during Dox induction.

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et al., 2001), we found that, like cyclin A, cyclin B can interact with HJURP (Figure S3B). However, this interaction is not dependent on an intact CD within HJURP, indicating inhibitory control in mitosis is exerted through a different mechanism.

Our mapping of the principal cyclin A interaction site on HJURP allowed us to determine the consequences of the loss of this interaction for the timing of its localization along the cell cycle. Upon removal of soluble HJURP by pre-extraction, we revealed that the stably chromatin-bound pre-mitotic HJURP-ΔCLacI was enriched in nucleoli (as observed previously by Dunleavy et al., 2009). In contrast, HJURP^{AxA}-ΔCLacI targeted to centromeres prematurely in the G2 phase, the time of the cell cycle in which cyclin A is the principal cyclin (Figure 2A). In addition, we analyzed CENP-A deposition using a SNAP tag-based fluorescent quench-chase-pulse labeling protocol that we described previously (Figure 2B) (Bodor et al., 2012; Silva et al., 2012). Remarkably, expression of the cyclin A binding mutant of HJURP, but not its wild-type counterpart, resulted in a precocious deposition of nascent CENP-A in the G2 phase. We performed these experiments using HJURP-ΔCLacI to force homodimerization of HJURP^{AxA}. In this way, we showed that HJURP^{AxA} itself is a functional assembly factor, independent of wild-type HJURP copies. Consistent with this, downregulation of endogenous HJURP showed no effect on either the efficiency or the frequency of premature CENP-A loading following GFP-HJURP^{AxA}-ΔCLacI expression (Figures S4A and S4B). Either GFP-HJURP^{AxA}-ΔCLacI (Figure 2B) or GFP-HJURP^{AxA} (carrying the endogenous C-terminal HJURP dimerization domain) (Figures 2B″ and S4C) expression result in a similar level of precocious deposition of CENP-A, demonstrating that uncoupling is not an artifact of LacI-mediated dimerization. Quantitative analysis showed that precocious CENP-A assembly at the centromere reached ~40% of the G1 levels (Figure 2B; see also Figure S4D) (i.e., 20% assembly per centromere, considering the replicated state of sister centromeres in the G2 phase, unresolvable by microscopy). We conclude that the CD of HJURP is a cell-cycle control element that interacts with cyclin A. Disruption of this site is sufficient to alleviate at least part of the Cdk-mediated inhibition of HJURP.

Cdk Activity Controls HJURP Localization, Not Its Chaperoning Activity

Phosphorylation of HJURP could directly interfere with its chaperoning activity, thereby inactivating the key function of the protein. Alternatively, it may sequester an otherwise active HJURP away from the centromere, preventing its untimely recruitment. To distinguish between these possibilities, we fused HJURP to the DNA-binding domain of CENP-B (CBdbd) (Figure 2C). This domain binds specifically to centromeric α-satellite DNA and allowed us to drive HJURP to centromeres in G2-synchronized cells, while likely bearing inhibitory phosphorylation due to high Cdk activity. We detected nascent CENP-A-SNAP at G2 centromeres after expression of HJURP-CBdbd-GFP (Figures 2C and 2D), but not CBdbd-GFP alone, indicating centromeric localization of HJURP is sufficient to enable unscheduled CENP-A loading. Although HJURP is removed from mitotic chromatin (a process that apparently overrides the DNA binding activity of the CENP-B DNA binding domain), newly loaded CENP-A-SNAP remained associated with centromeres upon entry into mitosis, suggesting it is assembled into centromeric nucleosomes rather than part of an HJURP-associated prenucleosomal complex (Figure 2C, right). Based on these results, we conclude that Cdk-driven phosphorylation does not interfere with HJURP chaperoning activity; rather, it results in sequestering HJURP away from the centromere, preventing its untimely recruitment.

HJURP Serine 210/211 Is Functionally Phosphorylated in G2 Phase Cells

Next, we determined whether the uncoupling of HJURP from its cell-cycle control involves specific phosphorylation sites. Expression of HJURP in which the six identified putative mitotic Cdk phospho-residues (Figure 1C) were mutated to alanine (either all six or combinations thereof) did not result in changes in the timing of CENP-A assembly (Figure S5B), despite previous reports implicating three of these residues (S412, S448, and S472; Müller et al., 2014). Because we observed premature CENP-A assembly in the G2 phase, during which cyclin A is the major cyclin, we aimed to identify additional potentially relevant phospho-residues in this cell-cycle window. We expressed doxycycline (Dox)-inducible 3×Flag-HJURP-ΔCLacI or 3×Flag-HJURP^{AxA}-ΔCLacI in the G2 phase-enriched HeLa high-efficiency and low-background (HILO) cells (Khandelia et al., 2011) (Figures 3A and 3B; see also the section below). Following 3×Flag-HJURP-ΔCLacI immunoprecipitation, titanium dioxide (TiO_2) phospho-enrichment, and mass spectrometry (Figure 3C), we identified S210/S211 phosphopeptides (the proximity of these residues prevented us from differentiating S210 versus S211 as the site of phosphorylation). These phosphopeptides

(C) Cell pellets obtained from the experiment in (B) were subjected to immunoprecipitation using Flag-coupled agarose beads to isolate 3×Flag-HJURP-ΔCLacI, separated on SDS-PAGE, and followed by Coomassie-based excision of HJURP proteins. Purified proteins were subjected to trypsin digestion and phosphopeptide enrichment, followed by liquid chromatography-mass spectrometry (LC-MS/MS) analysis.

(D) Mass spectra of a representative non-phosphorylated HJURP peptide from the flow through of the phospho-enrichment and of samples from cells containing WT HJURP-ΔCLacI (top) and HJURP^{AxA}-ΔCLacI (bottom).

(E) Mass spectra of the phosphopeptide containing pS210/pS211 from the elution of the phospho-enrichment from cells expressing indicated constructs. Because the two serines are adjacent, it was not possible to differentiate between S210 and S211 as the site of phosphorylation.

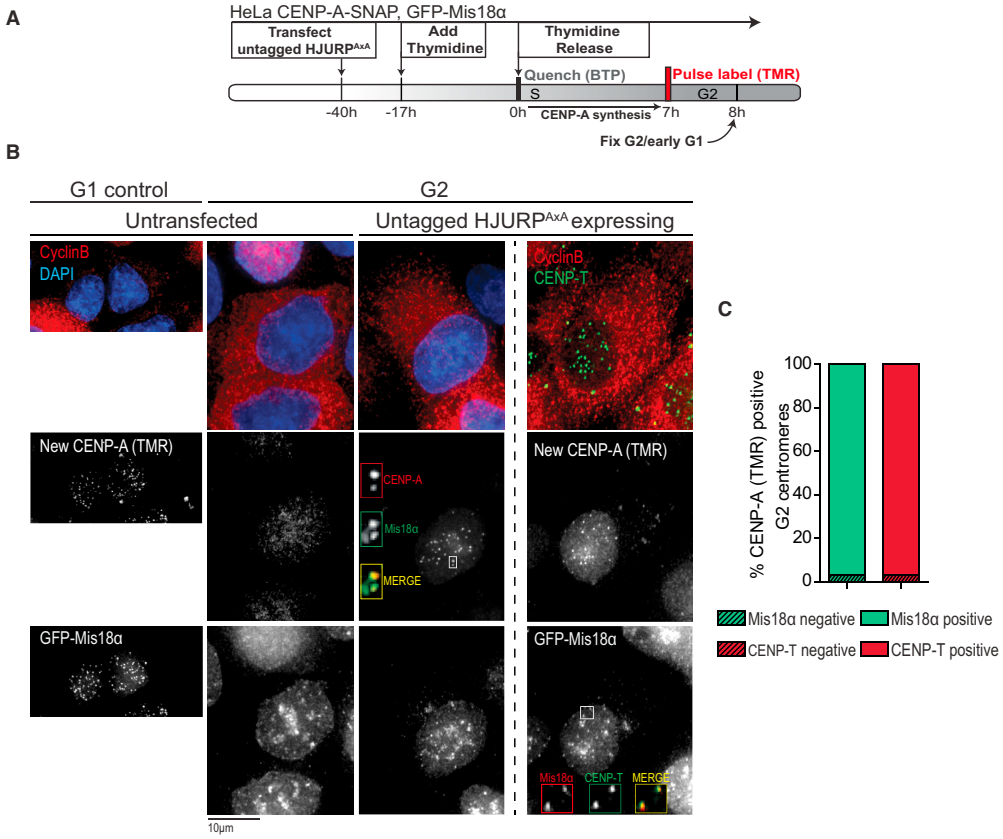
(F) Schematic representation of Cdk-consensus phospho-sites detected on HJURP in the G2 phase.

(G) Experiment analogous to Figure 2A assaying indicated HJURP constructs for localization and CENP-A assembly in the G2 phase.

(H) Representative images of cells from the experiment in (G). CENP-A assembly was assayed using SNAP TMR-labeling of its S-phase-synthesized pool. Following fixation, cells were counterstained for cyclin B and DAPI to indicate G2 status and DNA, respectively.

(H′) Left: quantification of frequency of premature CENP-A loading in cyclin B-positive cells expressing indicated constructs from three replicate experiments. Right: quantification of CENP-A-SNAP (TMR) fluorescent signal intensities.

See also Figure S5.



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were not detected in mitotically synchronized cells (Figure 1C), suggesting differential phosphorylation of HJURP, consistent with our finding that cyclin B also interacts with HJURP, but in a CD-independent manner (Figure S3B). Further, we found S412 to be the only common phospho-residue between G2 and mitotically synchronized cells (Figure 3F). Importantly, the relative abundance of S210/S211 phospho-peptides was substantially reduced on the HJURP^{AxA} mutant in which cyclin A binding was reduced compared to wild-type (Figure 3E). This suggests that the cyclin A/Cdk complex interaction with HJURP results in phosphorylation of this site.

To test the functional significance of these residues, we mutated serines 210 and 211 in combination with serine 412 and expressed HJURP^{S210A,S211A,S412A}-ΔCLacI mutants in the G2 phase cells. Quench-chase-pulse labeling of CENP-A-SNAP showed that mutation of these residues to alanine results in low, but detectable, levels of nascent CENP-A at centromeres (Figures 3G and 3H). This indicates that cyclin A binding to HJURP in the G2 phase results in phosphorylation, at least on serines S210/211 and S412, and that these modifications contribute to preventing premature CENP-A assembly.

HJURP^{AxA}-Induced CENP-A Assembly in G2 Phase Is Mis18 Dependent

Although HJURP^{AxA} is capable of inducing unscheduled CENP-A assembly, it does so with a relatively low efficiency and centromere specificity as compared to canonical G1 loading (Figures 2B′ and 2B″). This indicates that an additional level of cell-cycle control exists. A candidate for this is the Mis18 complex, which includes Mis18α, Mis18β, and the associated protein M18BP1 (Fujita et al., 2007). All subunits share a common localization pattern, with highly enriched and centromere-specific localization in anaphase, followed by disappearance in mid-G1 (Fujita et al., 2007; Silva and Jansen, 2009). Interestingly, we found that premature HJURP^{AxA} driven CENP-A assembly in the G2 phase correlates with low levels of stably expressed GFP-Mis18α at centromeres (Figures 4A–4C). Moreover, small interfering RNA (siRNA)-mediated depletion of Mis18α leads to a loss of both canonical assembly in the G1 phase as well as premature assembly of CENP-A in the G2 phase (Figure 4D). This demonstrates that HJURP^{AxA}-induced assembly occurs through the canonical assembly pathway and suggests that the partial nature of this assembly is possibly due to low levels of Mis18 complex members at G2 centromeres.

Recruitment of the Mis18 Complex to the Centromere Is Controlled by Phosphorylation of M18BP1^{T653}

Previously, we reported that a phospho-dead M18BP1 mutant, in which 24 known phospho-sites are mutated to alanine, resulted in its premature centromere targeting (Silva et al., 2012), suggesting that at least 1 of these sites is regulated by Cdks. We have now identified four putative Cdk motifs that are highly conserved among vertebrates, three of which are clustered close to the N terminus of M18BP1 (T4, T40, and S110), while a fourth (T653) is located between the highly conserved SANTA and SANT domains (Maddox et al., 2007) (Figure 5A). Mutation of all four sites to alanine leads to a loss of cell-cycle controlled localization of M18BP1 (Figure S6A). Interestingly, mutation of T653 alone was sufficient to result in premature centromere targeting of M18BP1, with an ~3-fold increase in centromeric levels relative to wild-type protein (Figure 5B). We generated a phospho- and site-specific antibody against the T653 site and show that pT653 levels rise as cells accumulate in S/G2 and mitosis, correlating with increasing levels of Cdk1 and -2 activities (Figure 5C). A brief treatment with the Cdk1/2 inhibitor of cells expressing GFP-M18BP1 caused a strong reduction in phosphorylation of T653, suggesting that M18BP1 is a direct target of these kinases (Figure 5D).

Further, the M18BP1^{T653A} mutant co-recruited Mis18α to G2 centromeres, indicative of ongoing Mis18 complex formation independent of T653 phosphorylation (Figure S6B). An N-terminal 490-amino acid fragment of M18BP1 was reported to be functional in supporting CENP-A assembly in the G1 phase (McKinley and Cheeseman, 2014), consistent with our finding that mutation of the T653 residue does not abrogate M18BP1 localization, but we now add that this residue controls cell-cycle dependent localization. To test whether M18BP1 phosphorylation of T653 results in disruption of the Mis18α interaction, we expressed a translational fusion of wild-type or mutant M18BP1 to the CBdbd in cells synchronized in the G2 phase (analogous to artificial HJURP tethering; Figure 2C). Forced recruitment of M18BP1 to centromeres leads to strong co-recruitment of Mis18α to G2 centromeres, suggesting that the Mis18 complex can form under inhibitory Cdk activity, at least at this stage in the cell cycle (Figure S6C), but not in mitosis, as observed previously (McKinley and Cheeseman, 2014). Similarly, forced recruitment of a phosphomimetic M18BP1^{T653D} (Figure S6C) or M18BP1^{T653E} mutant (data not shown) is capable of co-recruitment of Mis18α. Thus, we find that mutation of the T653 residue does not disrupt the M18BP1/Mis18α interaction. Rather, its phosphorylation prevents centromere targeting of the Mis18 complex in the G2 phase until mitotic exit, when Cdk1/2 activities are low.

Cdk-Mediated Control of M18BP1 and HJURP Is Sufficient to Ensure Tight Cell-Cycle Timing of Centromere Propagation

Our results indicate that centromere localization of both HJURP and M18BP1 is blocked by Cdk-mediated phosphorylation,

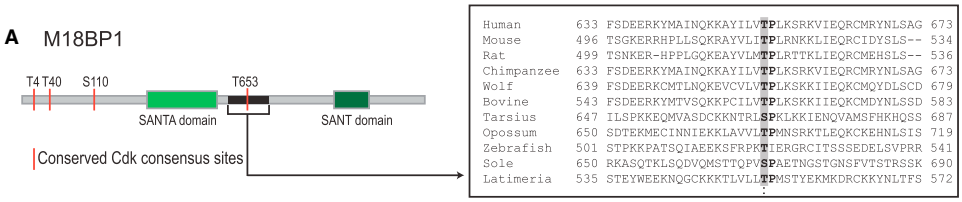
Figure 4. HJURP^{AxA}-Induced CENP-A Assembly Is Mis18α Dependent

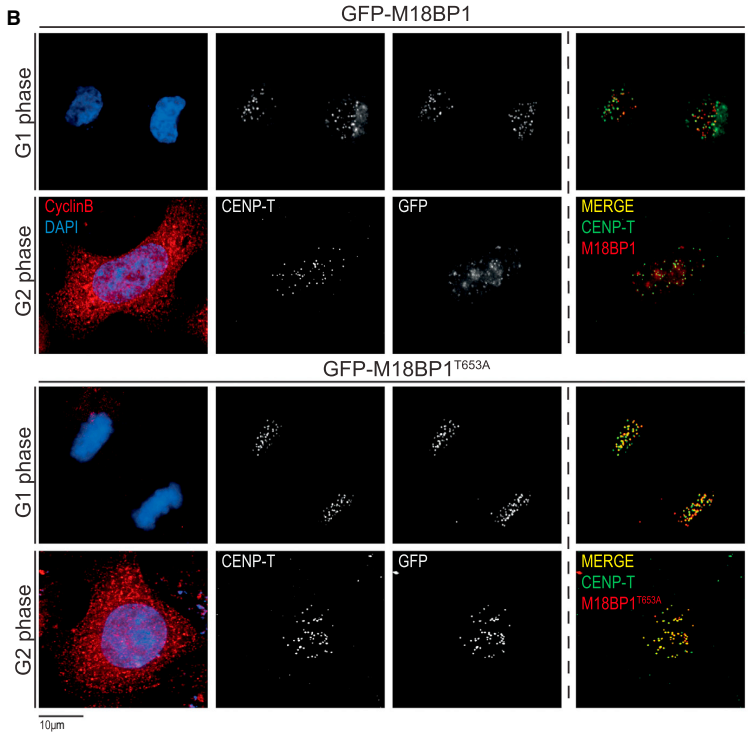
(A) Stable GFP-Mis18α, CENP-A-SNAP double transgenic HeLa cells were transfected with untagged HJURP^{AxA}, synchronized, and assayed for nascent CENP-A assembly by SNAP quench-chase-pulse labeling, followed by immunostaining for cyclin B and DAPI to indicate G2 status and DNA, respectively.

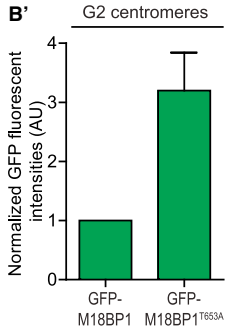
(B) Representative images of experiment described in (A).

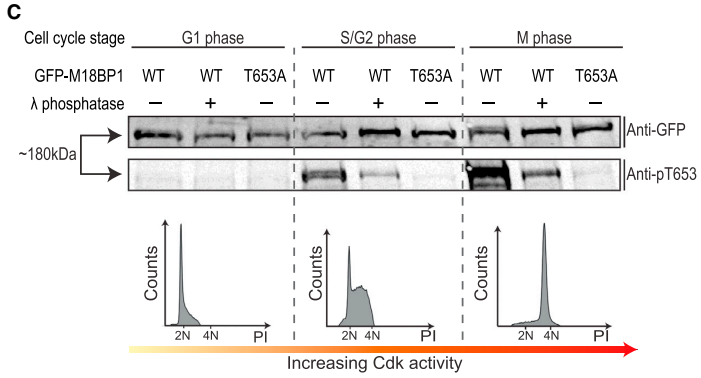
(C) Quantification of frequency of CENP-A (TMR)-positive G2 centromeres of the experiment described in (A). Cells were scored in relation to whether GFP-M18α (green) or CENP-T (red) signals were simultaneously detected together with CENP-A (TMR) or not.

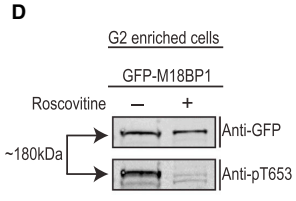
(D) Top: scheme outlining RNAi against Mis18α or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), synchronization, and quench-chase-pulse labeling of CENP-A-SNAP, GFP-Mis18α cells. Bottom: quantification of CENP-A-SNAP (TMR)-positive cells from three independent experiments. Error bars indicate SEM.











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suggesting that combined phospho-control of these protein complexes contributes to cell-cycle-specific loading of CENP-A. To directly test this, we constructed HeLa HILO cells expressing equal levels of either HJURP-ΔCLacI or HJURP^{AxA}-ΔCLacI (Figure S7A) under the control of a doxycycline-inducible promoter at a defined locus using recombination-mediated cassette exchange (RMCE) (Khandelia et al., 2011). HJURP induction was performed in cells either stably expressing GFP-tagged M18BP1^{T653A} or expressing endogenous M18BP1 along with CENP-A-SNAP to assay for CENP-A assembly (Figure 6A). We compared the efficiency of G2 phase loading to the normal level of assembly in the G1 phase. CENP-A assembly in uninduced control G1 cells was equal across all cell lines and essentially completed at the time of fixation (Figures S7B and S7C, respectively). As observed after transient expression, induction of HJURP^{AxA} alone resulted in low levels (~20% of G1, when corrected for centromere replication in G2 phase) of CENP-A assembly (Figures 6B, 6C, and S7D). Forced expression of otherwise wild-type but GFP-tagged M18BP1 does not enhance the degree of premature CENP-A assembly (Figures S7E and 7E′). Conversely, constitutive M18BP1^{T653A} expression led to infrequent and inefficient recruitment of nascent CENP-A to G2 centromeres (Figure 6C). Remarkably, induction of HJURP^{AxA} combined with stably expressed M18BP1^{T653A} resulted in highly efficient and centromere-restricted CENP-A assembly in the G2 phase, reaching 93% of G1 control levels (Figure 6C). In sum, disrupting the timing of centromere targeting of either HJURP or M18BP1 results in a limited deregulation of CENP-A assembly, as has been shown previously (McKinley and Cheeseman, 2014; Müller et al., 2014). We now show that simultaneous uncoupling of both of these proteins leads to full-fledged CENP-A assembly, indistinguishable from canonical G1 phase assembly. These findings strongly suggest that M18BP1 and HJURP are the two principal targets of Cdk-mediated inhibition.

Efficient CENP-A Assembly Requires Displacement of M18BP1 from the Centromere

During the course of these experiments, we observed that induction of CENP-A assembly in the G2 phase resulted in concomitant loss of centromeric GFP-M18BP1^{T653A} levels to <30%, on average, relative to the uninduced control (Figures 6C and 6D). Expression of HJURP^{AxA}-ΔCLacI, but not wild-type HJURP, results in GFP-M18BP1^{T653A} loss, showing that displacement is directly dependent on CENP-A assembly. This suggests that M18BP1 removal is an active CENP-A loading-dependent process and not a passive consequence of cell-cycle progression. To test this directly in G1 cells, we either overexpressed wild-type M18BP1 or artificially tethered it to G1 centromeres (using the CBdbd tether) while measuring nascent CENP-A chromatin assembly (Figures 7A and 7B). We observed an ~40% reduction in nascent CENP-A fluorescent intensities in both of these conditions (Figure 7C). We conclude that, while M18BP1 is an essential positive regulator of CENP-A assembly, preventing its turnover by overexpression or by rendering it unable to be removed from G1 centromeres results in defects in CENP-A assembly.

DISCUSSION

We have identified the licensing factor M18BP1 and the CENP-A chaperone HJURP as the two key targets of Cdk-based inhibition sufficient for maintenance of strict cell-cycle control of CENP-A assembly (Figure 7D). However, we do not exclude that additional levels of regulation exists (e.g., in chromatin maturation steps or in mitotic inhibition, which we find to be controlled in a distinct manner from the G2 phase).

Inhibition of CENP-A assembly prior to mitosis at the level of HJURP or M18BP1 alone is incomplete. This is in agreement with previous studies that showed that mutation of HJURP phospho-sites within the HJURP C-terminal domain 1 (HCTD1) (Müller et al., 2014) or forced recruitment of Mis18α resulted in precocious CENP-A assembly (McKinley and Cheeseman, 2014). We note that, in our system, mutation of the HCTD1 phosphosites did not result in precocious CENP-A assembly (Figure S5B). This discrepancy is likely the result of expression level differences between the cell types used in each study (Figure S5C and Bodor et al., 2014).

We provide evidence that the primary mechanism of Cdk-mediated inhibition is to prevent otherwise active factors from reaching the centromere (Figures 2C, S6B, S6C and S7D). We propose that phosphorylation blocks the ability of M18BP1 and HJURP to bind to a partner(s) already docked at the centromere. M18BP1 interacts with CENP-C, which is a constitutive core component of the centromere (Dambacher et al., 2012; Hori

Figure 5. Cdk-Mediated T653 Phosphorylation of M18BP1 Controls Its Centromere Recruitment

(A) M18BP1 T653 is conserved among vertebrates. Left: schematic of M18BP1 protein. Relevant domains and conserved Cdk sites are indicated. Right: conservation of the human T653 residue across species. Conserved threonine or serine is highlighted in gray.

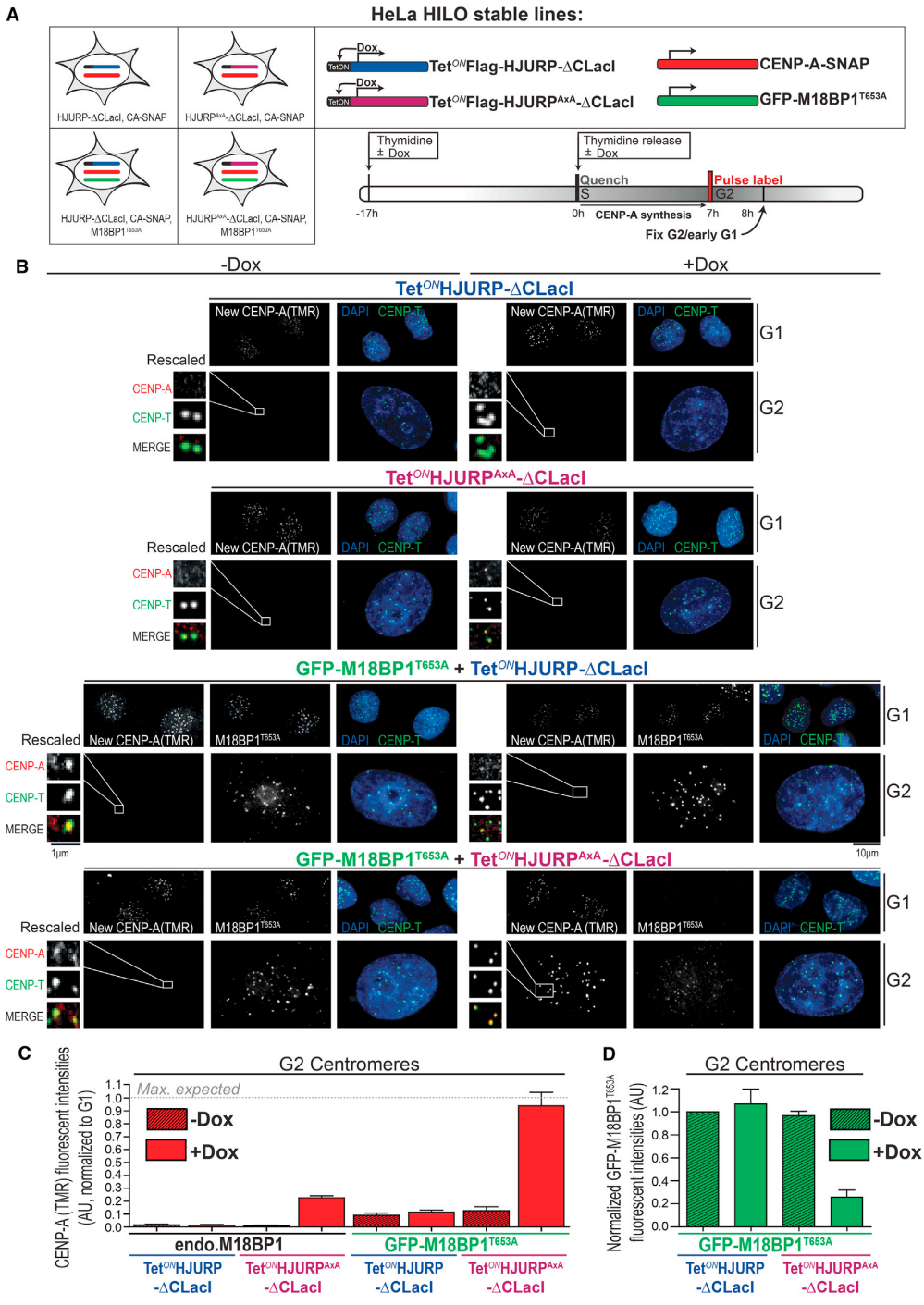
(B) T653 residue controls cell-cycle-dependent M18BP1 centromere recruitment. Indicated constructs were transfected into asynchronous HeLa cells 48 hr prior to fixation, followed by counterstaining for cyclin B, CENP-T, and DAPI to indicate G2 status, centromeres, and DNA, respectively.

(B′) Average centromeric GFP fluorescent signals from cyclin B-positive cells were determined using the centromere recognition and quantification (CRaQ) method (Bodor et al., 2012) and normalized to GFP-M18BP1. Error bars indicate SEM from three replicates.

(C) T653 is phosphorylated in a cell-cycle-dependent manner. HEK293T cells were transiently transfected with GFP-Mis18BP1 (wild-type [WT]) or GFP-Mis18BP1^{T653A} as a non-phosphorylatable control. 24 hr later, cells were synchronized in indicated cell-cycle stages and lysed. Extracts were either left untreated or treated with lambda phosphatase, separated by SDS-PAGE, and followed by immunoblotting with indicated antibodies (see also Supplemental Experimental Procedures). Apparent molecular weight is indicated. Cells were assayed for cell-cycle position by fluorescence-activated cell sorting (FACS) using propidium iodide (PI) to indicate DNA content.

(D) T653 is phosphorylated by Cdk1/2. HEK293T cells were transiently transfected with GFP-Mis18BP1 and enriched in the G2 phase by a single thymidine block, followed by 7 hr of release. 30 min before fixation, cells were treated with 100 μM Roscovitine. Extracts were separated by SDS-PAGE followed by immunoblotting with anti-GFP and anti-pT653 antibodies.

See also Figure S6.



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et al., 2013; Moree et al., 2011; Nardi et al., 2016; Stellfox et al., 2016). In turn, the prenucleosomal HJURP/CENP-A complex binds to the Mis18 complex (Nardi et al., 2016; Wang et al., 2014). Our proposal is consistent with a recent report describing an interaction between HJURP and the Mis18 complex subunit Mis18β, which is reduced upon Cdk phosphorylation in vitro (Wang et al., 2014).

Recent studies have reported cell-cycle-regulated phosphorylation of CENP-A itself (Yu et al., 2015) or Plk1-mediated modification of M18BP1 (McKinley and Cheeseman, 2014). Although the latter is required for Mis18 complex localization upon mitotic exit, none of these modifications directly dictates the G1-restricted CENP-A assembly. Therefore, while key positive regulatory events also involve phospho-regulation (which may include some of the novel phosphorylation sites that we identified on HJURP), we defined the specific targets and mechanisms of the inhibitory control that is responsible for limiting CENP-A assembly to the G1 phase. Rather than relying on a single tightly regulated factor, the combinatorial action of two layers of control synergizes to efficiently restrict CENP-A assembly to the early G1 phase.

The designation of the Mis18 complex as a priming (licensing) factor was originally inspired by its temporal centromere localization that initiates in anaphase, before the onset of CENP-A assembly (Fujita et al., 2007). This is analogous to the licensing of DNA replication by the assembly of the pre-replication complex (pre-RC) in the early G1 phase (Nishitani and Lygerou, 2002), the S-phase removal of which ensures a single round of genome duplication per cell cycle (Blow and Dutta, 2005; Blow and Hodgson, 2002). Analogously, we find that removal of M18BP1 from the centromere is directly coupled to the onset of CENP-A deposition, at least under induced conditions in the G2 phase, providing a causal link between efficient CENP-A assembly and M18BP1 displacement from the centromere.

These results reveal novel parallels between DNA replication and CENP-A-chromatin, manifested in consumption of the licensing factor, which is directly instigated by the start of duplication of the heritable mark. These findings are consistent with a recent study showing that nascent CENP-A/HJURP binding to the Mis18 complex in vitro leads to the disassembly of this complex (Nardi et al., 2016), suggesting that Mis18 complex disassembly could be a mechanism to turn off CENP-A chromatin assembly. We show that CENP-A assembly not only results in Mis18 complex removal (as shown by Nardi et al., 2016), but that this is a requirement for efficient loading of CENP-A. Two possible implications follow from these observations. First, while M18BP1 is required for recruitment of nascent CENP-A to centromeres, its presence may physically block completion of the assembly process. By direct binding to CENP-C (Dambacher et al., 2012; Moree et al., 2011; Shono et al., 2015; Westhorpe et al., 2015), which in turn interacts with CENP-A (Falk et al., 2015; Guse et al., 2011; Kato et al., 2013; Logsdon et al., 2015), it is possible that M18BP1 physically marks the site of incorporation for nascent CENP-A. The inability to remove M18BP1 would therefore provoke steric inhibition, resulting in low rates of CENP-A incorporation. Second, given the key role in initiation of CENP-A loading, removal of M18BP1 from centromeres provides an "OFF" switch for the process of assembly, thereby contributing to a tight cell-cycle window, ensuring a single round of CENP-A incorporation per cell cycle.

EXPERIMENTAL PROCEDURES

Cell Synchronization

Double thymidine-based synchronization was performed as described (Bodor et al., 2012). For mitotic synchronization, 2.4 μM of EG5 inhibitor III Dimethylenastron-DMEIII (Calbiochem) was used for 24 hr. For synchronous mitotic exit, following DMEIII washout, HeLa and HEK293T cells were released for 5 hr and 7 hr, respectively. For Figure 2C, nocodazole was used at 100 ng/mL.

Co-immunoprecipitation

HEK293T cells were transiently transfected with GFP-tagged constructs. 24 hr post-transfection, cells were either allowed to continue to cycle or were treated overnight in DME III to induce mitotic arrest. Cells were harvested 48 hr post-transfection and subjected to cyclin A or B immunoprecipitation (see also Supplemental Experimental Procedures). Isolated complexes were separated by SDS-PAGE, followed by immunoblotting with anti-cyclin A (Figure 1E) or anti-cyclin B (both Santa Cruz) (Figure S3B) and anti-GFP (Chromotek) antibodies, detected on an Odyssey near-infrared scanner, and quantified using the Odyssey software (see also Bodor et al., 2014).

SILAC and Affinity Purification of Prenucleosomal HJURP/CENP-A/H4 Complex

SILAC-labeling medium was supplemented with normal lysine and arginine (Sigma-Aldrich) for "light" medium, and 50 mg/L $^{13}C_6$,$^{15}N_2$-lysine and 50 mg/L $^{13}C_6$,$^{15}N_4$-arginine (Silantes) for "heavy" medium (see also Supplemental Experimental Procedures). Two parallel cultures of previously characterized HeLaS3 cells stably expressing (LAP)-tagged CENP-A (Bailey et al., 2013) were grown in either heavy or light medium until reaching ~98% labeling efficiency. To enrich for mitotic cells, both cultures were treated with 50 μM S-trityl-L-cysteine for 17 hr. Subsequently, the "light" cells were treated with 100 μM R-Roscovitine (AdipoGen) for 30 min while the "heavy" cells

Figure 6. A Dual Inhibitory Mechanism Restricts CENP-A Deposition to G1 Phase

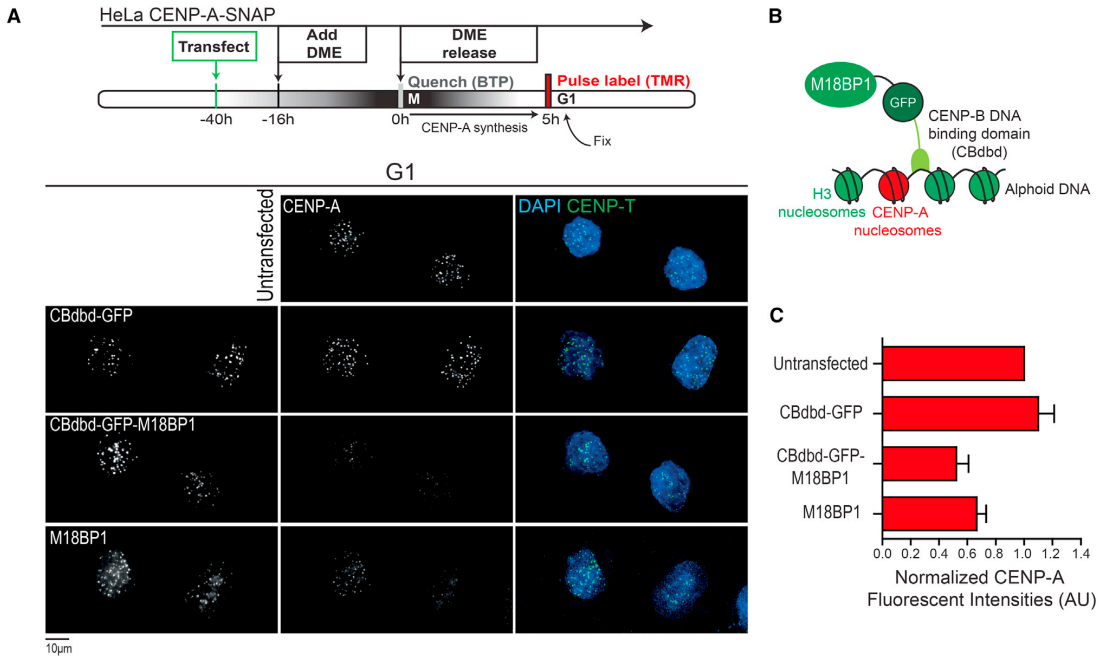
(A) Schematic representation of HeLa HILO cells carrying low levels of constitutively expressed CENP-A-SNAP (red) with or without stable expression of GFP M18BP1^{T653A} (green), along with doxycycline-inducible 3×Flag-HJURP-ΔCLacI (blue) or 3×Flag-HJURP^{AxA}-ΔCLacI (purple). Cells were processed as indicated in the scheme.

(B) Representative images of the experiment described above. Following fixation, cells were counterstained for CENP-T and DAPI to indicate centromeres and DNA, respectively. Cell-cycle status was determined by measuring total DAPI area (see Supplemental Experimental Procedures).

(C) Quantification of CENP-A-SNAP fluorescent signals from (B). Average CENP-A-SNAP signals from G2 centromeres were normalized to respective G1 centromeres and corrected for centromere number (assuming signal intensity per focus represents one and two centromeres in G1 and G2, respectively). Error bars indicate SEM of four independent experiments.

(D) CENP-A assembly drives M18BP1 displacement from centromeres. Quantification of centromeric GFP-M18BP1^{T653A} fluorescent signals from (B) using CRaQ method. Average GFP-M18BP1^{T653A} signals were normalized to uninduced 3×Flag-HJURP-ΔCLacI-expressing cells. Error bars indicate SEM of four independent experiments.

See also Figure S7.



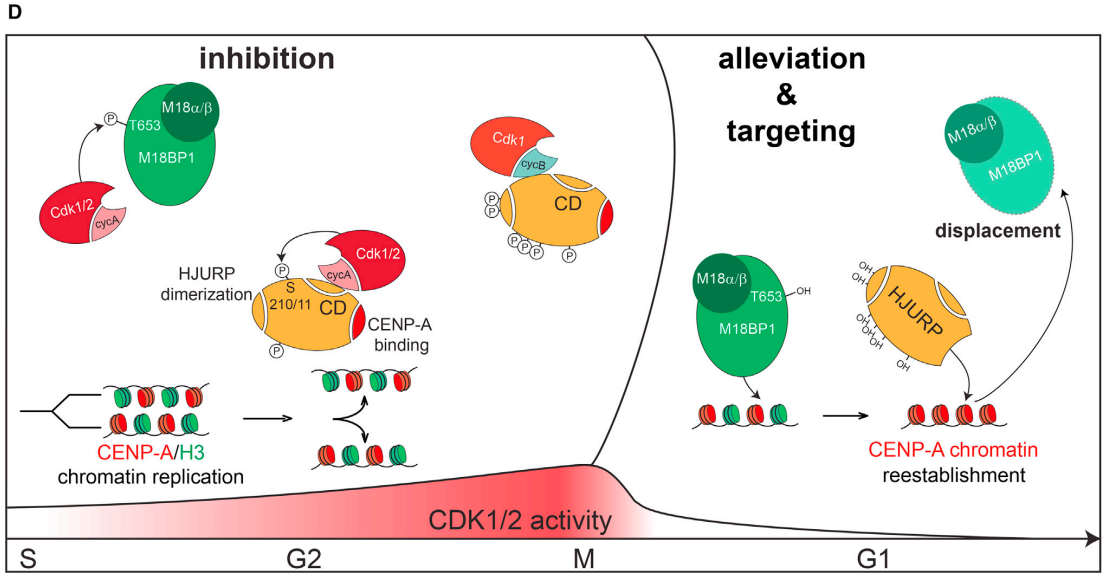


Figure 7. M18BP1 Removal from G1 Centromeres Is Necessary for Efficient Canonical CENP-A Assembly

(A) HeLa CENP-A-SNAP cells were transfected with indicated constructs and synchronized in mitosis by an overnight treatment with Eg5 inhibitor (DMEIII). Newly synthesized CENP-A pool was quenched in mitosis, followed by 5 hr of release in early G1 when nascent CENP-A-SNAP was labeled with TMR (G1-specific pool).

(legend continued on next page)

were mock-treated with DMSO. Affinity purification of the prenucleosomal HJURP/CENP-A/H4 complexes from 1:1 mixed "light" and "heavy" cells was performed as previously described (Bailey et al., 2013) except that protein elution was performed with 2% SDS and heating at 95°C.

Mass Spectrometry and Data Analysis

Purified CENP-A and associated proteins were precipitated, washed, and dried. Following reconstitution, proteins were cleaved with trypsin and phosphopeptides and enriched by TiO_2 prior to mass spectrometry analysis (see Supplemental Experimental Procedures for details). Extracted-ion chromatograms (XICs) of each light and heavy peptide pair were used for quantification. The light/heavy (L/H) ratio represents the ratio of total area under each elution peak.

Affinity Purification of 3XFlag-HJURP$^{wt/AxA}$-ΔCLacI and Mass Spectrometry

HeLa HILO RMCE cell lines carrying either 3×Flag-HJURP-ΔCLacI or 3×Flag-HJURP^{AxA}-ΔCLacI were enriched in the G2 phase as described (see Cell Synchronization) and induced with 10 μg/mL of doxycycline (Sigma) for 24 hr. HJURP was affinity purified using anti-Flag M2 mouse agarose beads (Sigma-Aldrich) as described (see Co-immunoprecipitation), followed by SDS-PAGE separation of bound complexes, staining (by Instant Blue, Expedeon), and subsequent HJURP band excision in-gel trypsin digestion and phosphopeptide enrichment by TiO_2. Samples were run on a Q Exactive mass spectrometer coupled with Easy nLC 1000 HPLC. MaxQuant was used to search the human protein database, identify peptide sequences, and extract their ion chromatograms.

SNAP Quench-Chase-Pulse Labeling

Cell lines expressing CENP-A-SNAP were pulse labeled as previously described (Bodor et al., 2012), with the exception of HeLa HILO-derived cell lines, where bromothenylpteridine (BTP; New England Biolabs) concentration was adjusted to 0.5 μM.

Immunofluorescence and Pre-extraction Procedure

Procedures are essentially as described (Bodor et al., 2012) (see also Supplemental Experimental Procedures). To detect GFP-HJURP^{AxA}-ΔCLacI on G2 centromeres, HeLa CENP-A-SNAP cells transiently expressing the construct were pre-extracted for 5 min prior to fixation. Cells were counterstained using anti-CENP-T (Barnhart et al., 2011) and anti-Aurora B (1:100; BD Transduction Laboratories). GFP-HJURP^{AxA}-ΔCLacI signal was amplified using GFP-Booster Atto488 (Chromotek).

Microscopy

Imaging was performed using a DeltaVision Core system (Applied Precision) inverted microscope (Olympus, IX-71) coupled to a Cascade2 EMCCD camera (Photometrics). Images (1024 × 1024) were acquired at 1× binning using a 100× oil objective (NA 1.40, UPlanSApo) with 0.2 μm z sections.

ACCESSION NUMBERS

Unprocessed image data used to prepare the figures in this manuscript have been deposited in Mendeley Data and are available at http://dx.doi.org/10.17632/8833hjzmnf.1.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2016.11.021.

AUTHOR CONTRIBUTIONS

A.S. performed the experiments. L.Y.G. performed CENP-A/HJURP co-purifications for SILAC experiments and helped write the manuscript. J.F.M. generated constructs and performed experiments in Figure 4. D.L.B. generated constructs for experiments in Figures 6 and S7. A.O.B., J.S., and D.F.H. performed the initial mass spectrometry analysis of HJURP phosphorylation sites. L.Y.G., X.-J.C., B.E.B., and B.A.G. performed mass spectrometry experiments and analysis. L.E.T.J. and B.E.B. directed the research. B.E.B. helped write the manuscript. A.S. and L.E.T.J. conceived of the study and wrote the manuscript.

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(B) Schematic of relevant domains in centromere-targeted M18BP1.
(C) GFP-positive cells were selected and CENP-A TMR fluorescent intensities were determined using CRaQ, with the exception of the untransfected control, where all cells were analyzed.
(D) Model summarizing the key molecular steps that are sufficient to restrict CENP-A assembly to the G1 phase. CD: HJURP vertebrate-conserved domain.

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